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Some problems encountered in attempting to estimate catecholamine turnover using labelled tyrosine

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Rats were given [^{3}H]tyrosine intravenously. The decrease of [^{3}H]noradrenaline (^{3}H-NA) and [^{3}H]dopamine (^{3}H-DA) from 2 to 6 h after the [^{3}H]tyrosine injection was estimated in the cerebral cortex, brain stem, caudate nucleus, spinal cord and adrenal medulla. Half-lives were estimated with or without the tyrosine hydroxylase inhibitor H 44/68 or the dopamine-β-hydroxylase inhibitor FLA-63, given 2 h after the [^{3}H]tyrosine. The influence of the inhibitors on endogenous noradrenaline was also studied. H 44/68 did not change the half-life of ^{3}H-NA in the central nervous system but FLA-63 reduced it considerably. FLA-63 reduced endogenous noradrenaline more than did H 44/68. The mechanism responsible for the faster turnover of noradrenaline after FLA-63 has not yet been elucidated. In the adrenal medulla the [^{3}H]adrenaline + ^{3}H-NA level rose significantly from 2 to 6 h after the injection of [^{3}H]tyrosine. The synthesis inhibitors prevented this rise, indicating that appreciable amounts of labelled precursors remain for a long time after the injection of the labelled tyrosine. Some problems encountered with the different methods for turnover determinations are discussed on the basis of these data.

Several methods have been used to estimate the turnover of catecholamines. For example, the rate of disappearance of a labelled amine after either administration of the amine itself (Brodie, Costa & others, 1966; Costa & Neff, 1966) or of a labelled precursor (Udenfriend & Zaltman-Nirenberg, 1963; Burack & Draskóczy, 1964; Nybäck, Borzecki & Sedvall, 1968; Sedvall, Weise & Kopin, 1968) has been estimated. Another approach has been to estimate the rate of disappearance of the endogenous amines after synthesis inhibition (Brodie & others, 1966; Costa & Neff, 1966; Andén, Corrodi & Fuxe, 1969; Neff, Ngai & others, 1969). Other methods have been used like measuring the accumulation of labelled amines after infusion or injection of labelled tyrosine (Gordon, Spector & others, 1966; Neff & others, 1969) or changes in the levels of the catecholamine metabolites (c.f. Carlsson & Lindqvist, 1963; Andén Roos & Werdinius, 1964; Sharman, 1969; Guldberg, 1969).

In a previous publication (Persson, 1969), turnover estimations using tritium-labelled tyrosine or dopa of high specific activity, were made and discussed. In the heart, the half-lives of the catecholamines were long in comparison to the times obtained after administration of the labelled amine itself. One explanation of this may be the difficulty in separating the synthesis and elimination processes from each other. Refilling of the labelled amine stores may occur from remaining labelled precursors and that will result in an apparently delayed disappearance. This

methodological problem may be circumvented by giving a synthesis inhibitor at a suitable time interval to stop the refilling. In the present investigation a tyrosine hydroxylase inhibitor (H 44/68) and a dopamine-β-hydroxylase inhibitor (FLA-63) have been used for this purpose.

EXPERIMENTAL

Male albino rats, 150–200 g, were used; all experiments were made at an ambient temperature of 30°. No hypothermia occurred when the synthesis inhibitors were given. The following drugs were used: L-tyrosine, ring 3,5-^{3}H (specific activity 36 Ci/mmol), the inhibitor of dopamine-β-hydroxylase, FLA-63 [bis(4-methyl-1-homopiperazinylthiocarbonyl) disulphide, Svensson & Waldeck, 1969; Carlsson, Corrodi, Florvall, Ross & Sjöberg, to be published] and the methylester of α-methyl-tyrosine (H 44/68), which inhibits the tyrosine hydroxylase (cf. Corrodi & Hanson, 1966). The brain was dissected as described by Persson (1969) and the cerebral cortex, brain stem, caudate nucleus, spinal cord and adrenal medulla analysed. Analyses were made on tissues collected and pooled from two rats. ^{3}H-NA and ^{3}H-DA were measured as described by Persson & Waldeck (1968). The ^{3}H-NA fraction from the adrenal medulla also contained [^{3}H]adrenaline (^{3}H-A) (Häggendal, 1962).

The isotope substances were obtained from The Radiochemical Centre, Amersham, England.

RESULTS

Rats received 5 μg/kg of [^{3}H]tyrosine intravenously and 2 h later 40 mg/kg of FLA-63 or 250 mg/kg of H 44/68 intraperitoneally. After another 4 h the animals were killed and the [^{3}H]catecholamines in the various tissues determined. Control animals received only [^{3}H]tyrosine and were killed 2 or 6 h later. In analogous experiments the endogenous noradrenaline levels were determined 4 h after administration of the synthesis inhibitors.

Effect of FLA-63 and H 44/68 on the disappearance of ^{3}H-NA, ^{3}H-DA and endogenous noradrenaline in different parts of the brain.

The results are presented as the mean values $\pm$ s.e. The apparent half-lives were calculated by regression analysis, assuming that the disappearance of the amines was a single exponential process (Table 1).

The results show that H 44/68 probably did not change the rate of disappearance of ^{3}H-NA. In the caudate nucleus the half-life of ^{3}H-DA was estimated to be about 3·5 h and this was reduced to 2 h after H 44/68 but the difference was not statistically significant. FLA-63 enhanced the disappearance rate of ^{3}H-NA significantly in the brain stem ($P < 0{\cdot}001$) and in the spinal cord ($P < 0{\cdot}05$). In the cortex there was a similar difference, though this was not significant. FLA-63 did not change the rate of disappearance of ^{3}H-DA in the caudate nucleus. After administration of H 44/68 or FLA-63 the endogenous noradrenaline levels decreased at about the same rate (or possibly faster) than did the ^{3}H-NA (Table 2).

The ^{3}H-DA levels in cortex, brain stem and the spinal cord were estimated but because of a relatively large scatter the data have been omitted. ^{3}H-NA in the caudate nucleus was also determined, but the large excess of ^{3}H-DA in this tissue made these results uncertain.

Table 1. *Effect of FLA-63 and H 44/68 on the disappearance of [³H]noradrenaline formed from [³H]tyrosine in different parts of the CNS and of [³H]dopamine in the caudate nucleus of the rat.* [³H]Tyrosine, 5 μg/kg was given i.v. to rats and 2 h later FLA-63, 40 mg/kg or H 44/68, 250 mg/kg i.p. After another 4 h the animals were killed and the [³H]amine levels in the different parts of the CNS determined. Control animals received [³H]tyrosine and were killed 2 and 6 h later. Figures in brackets denote the number of experiments, each comprising two animals

Tissue		Control 2 h	Control 6 h	FLA-63	H 44/68
		[³H]noradrenaline, fmol/g, and apparent half lives h			
Cortex	mean	14·6 (6)	9·3 (5)	4·6 (4)	7·8 (4)
	s.e.	2·54	0·74	0·92	0·36
	T 1/2		7·3	2·4	5·0
Brain stem ..	mean	33·2 (5)	20·4 (6)	7·0 (4)	20·2 (5)
	s.e.	3·05	2·17	1·37	1·51
	T 1/2		5·5	1·7	5·6
Spinal cord ..	mean	17·4 (6)	9·7 (6)	4·6 (5)	9·9 (5)
	s.e.	1·26	0·59	0·54	0·62
	T 1/2		4·8	2·1	4·9
		[³H]dopamine, fmol/g, and apparent half lives h			
Caudate nucleus ..	mean	461 (6)	213 (6)	225 (5)	14 (5)
	s.e.	45	24	43	34
	T 1/2		3·5	3·6	2·0

fmol = 10^{-15} mol.

Table 2. *Effect of FLA-63 and H 44/68 on the disappearance of noradrenaline in different parts of the CNS of the rat.* FLA-63, 40 mg/kg or H 44/68, 250 mg/kg was given i.p. to rats. 4 h later the animals were killed and the different parts of the CNS determined for noradrenaline

Tissue		Noradrenaline, μg/g, and apparent half lives h Control	FLA-63	H 44/68
		n=4	n=5	n=5
Cortex	mean	0·24	0·013	0·10
	s.e.	0·012	0·005	0·005
	T 1/2		—	3·2
Brain stem	mean	0·65	0·090	0·39
	s.e.	0·048	0·015	0·037
	T 1/2		1·4	5·4
Spinal cord	mean	0·35	0·060	0·15
	s.e.	0·034	0·009	0·021
	T 1/2		1·5	3·1

Effect of FLA-63 and H 44/68 on the disappearance of [³H]catecholamines formed from [³H]tyrosine in the adrenal medulla of the rat

The ³H-NA + ³H-A of the adrenals increased from 2 to 6 h after the [³H]tyrosine injection in the absence of a synthesis inhibitor ($P < 0{\cdot}05$) (Fig. 1). FLA-63 and H 44/68 inhibited this increase ($P < 0{\cdot}001$ and $P < 0{\cdot}01$, respectively). The ratio ³H-DA/³H-NA rose after FLA-63. The ³H-DA levels of the adrenal medulla appeared to decrease, though not significantly, from 2 to 6 h in animals not given synthesis inhibitors ($0{\cdot}05 < P < 0{\cdot}10$). After FLA-63 treatment the ³H-DA level

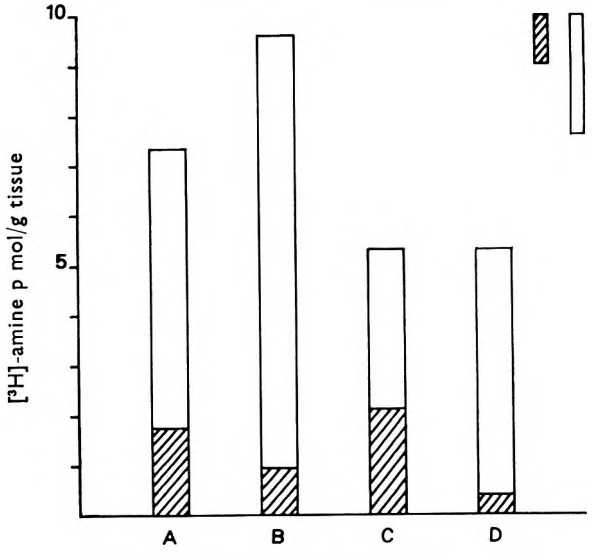

FIG. 1. Effect of FLA-63 and H 44/68 on the disappearance of [³H]catecholamines formed from [³H]tyrosine in the adrenal medulla of the rat. [³H]Tyrosine, 5 μg/kg was given i.v. to rats and 2 h later FLA-63, 40 mg/kg or H 44/68, 250 mg/kg, i.p. After another 4 h the animals were killed and [³H]noradrenaline + [³H]adrenaline and [³H]dopamine in the adrenals estimated. Control animals received [³H]tyrosine and were killed 2 and 6 h later. The means (based on 4—6 determinations) and differences ($P<0.05$) are given. Open columns: [³H]noradrenaline + [³H]adrenaline. Hatched columns [³H]dopamine. A: control 2 h, B: control 6 h, C: FLA-63, D: H 44/68.

was significantly higher than in the control; an opposite trend, though not significant, was observed after H 44/68.

DISCUSSION

Although much used for estimations of catecholamine formation and turnover, the isotope and synthesis inhibitor methods appear to require further analysis before their accuracy can be regarded as settled. Brodie & others (1966) reported a very good agreement for noradrenaline turnover rate in heart using an isotope method or a tyrosine hydroxylase inhibitor. On the other hand Westfall & Osada (1969) found obvious discrepancies with the above-mentioned methods and with measuring the conversion of labelled tyrosine to labelled noradrenaline in estimating the noradrenaline turnover in heart before and after adrenalectomy. With the central nervous system, only indirect comparisons can be made. Generally, a faster turnover of dopamine than of noradrenaline in brain is indicated, also some regional differences occur (see Persson, 1969). The aim of the present study was to compare these two methods for the estimation of catecholamine turnover and to examine further the methodological problems associated with the isotope technique (see Introduction). We found that administration of a tyrosine hydroxylase inhibitor 2 h after the injection of [³H]tyrosine did not change the rate of disappearance of ³H-NA. Sedvall & Nybäck (personal communication) agree. This cannot, however, be taken as evidence that the isotope method gives a true indication of turnover. The reasons for this are evident from the following discussion.

Inhibition of tyrosine hydroxylase and of dopamine-β-hydroxylase induced different rates of noradrenaline depletion. The disappearance of both ³H-NA and

endogenous noradrenaline was more rapid when dopamine-β-hydroxylase was inhibited. Goldstein & Nakajima (1967) reported similar findings. In a preliminary report we (Persson & Waldeck, 1970) have presented some experimental support for the view that the activity of the noradrenaline-containing neurons is affected by the activity in the dopamine neurons. When both dopamine and noradrenaline syntheses are inhibited, the turnover of the latter amine appears to be slowed. When only the dopamine-β-hydroxylase is inhibited, the influence of the dopamine neurons on the noradrenaline neurons is undisturbed. Such a mechanism would explain the difference in action of H 44/68 and FLA-63 on ^{3}H-NA and endogenous noradrenaline depletion. Another possible mechanism might be an incomplete inhibition of noradrenaline synthesis by H 44/68 but a more complete inhibition by FLA-63. However, when H 44/68 or FLA-63 were injected into mice in the same doses as have been used here, the synthesis of ^{3}H-NA from [^{3}H]tyrosine was less than 10% of normal after 30 min (Svensson & Waldeck, 1969, and unpublished data). It is unlikely that FLA-63 has a releasing action of its own, since noradrenaline disappears very slowly in the transected spinal cord after injection of this drug (Andén, personal communication). Preliminary experiments with DL-^{3}H-NA do not indicate any releasing effects of FLA-63 from mouse heart [Persson & Waldeck, unpublished].

The tyrosine hydroxylase inhibition as a method for estimation of noradrenaline turnover therefore seems to have the disadvantage of slowing noradrenaline turnover. On the other hand, when isotope precursor methods are used the extent to which the disappearing isotope-labelled amines are replaced by remaining labelled precursors is not known. Both methods therefore indicate too slow a turnover of noradrenaline. Another possible source of error is an unequal labelling of storage pools, since much experimental work (Hillarp, 1960; Lundborg, 1963; Häggendal & Lindqvist, 1963; Andén & Henning, 1966; see Kopin, Breese & others, 1968) indicates the presence of a small functionally active pool with high turnover and a large pool from which the amines are slowly released. During the comparatively long time intervals used for estimating the disappearance of the labelled amines in these experiments, only the turnover of the large pool would then be reflected in the half-lives obtained with [^{3}H]tyrosine. The estimations of the catecholamine turnover in the brain with the present methods are therefore not very accurate.

There are no statistically significant differences in the turnover of ^{3}H-DA with or without the synthesis inhibitors. But the invalidations of the methods discussed above may also apply to the estimations of dopamine turnover. We have not found any indication that the dopamine neurons depend on the noradrenaline neurons.

In the adrenal medulla, [^{3}H]adrenaline + ^{3}H-NA increased rather than decreased between 2 and 6 h after the administration of [^{3}H]tyrosine. This may in part be due to conversion of intermediate ^{3}H-DA since this amine appeared to decrease during this time interval, a decrease which failed to occur when the dopamine-β-hydroxylase had been blocked. A supply of [^{3}H]tyrosine or [^{3}H]dopa, or both, during this time interval has also to be taken into account, since the total [^{3}H]catecholamine content was reduced after synthesis inhibition. Whether this probable supply of precursor comes from the blood stream or from stores in the adrenal medulla remains to be elucidated. In any event it would appear that the synthesis of ^{3}H-A + ^{3}H-NA from [^{3}H]tyrosine is not completed within 2 h of the injection and that any con-

clusions about the exact turnover rates of catecholamines from the present data are hazardous. According to Burack & Drascóczy (1964) who used [^{3}H]dopa as catecholamine precursor, and to Brodie & others (1966), who used a tyrosine hydroxylase inhibitor, the catecholamines in the adrenal medulla had a very slow turnover with half-lives of about a week.

It may be concluded that there are many uncertainties about catecholamine turnover estimations with the present methods. They are, however, valuable for comparative studies.

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The absorption and excretion of carbenoxolone in man

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Blood, urine and bile concentrations of carbenoxolone have been measured after oral dosage to patients. The compound is rapidly absorbed attaining high blood concentrations and, as with rats, is excreted mostly in the bile, with only traces (2% of the dose) appearing in the urine. Blood concentrations of the drug generally exhibit two maxima, at 1–2 and 3–6 h after dosage, which are taken to indicate enterohepatic circulation of the biliary-excreted conjugates. As absorption of orally administered carbenoxolone is so rapid and does not occur when the gastric contents have pH >2, it is inferred that the major site of absorption is the stomach. The high blood concentrations of carbenoxolone (60% of the dose present in the blood) are probably due to a high degree of binding of the drug to the plasma proteins. In contrast to the rat, in which carbenoxolone is largely hydrolysed to β-glycyrrhetic acid before absorption, in man carbenoxolone is absorbed largely unchanged and is excreted in the bile as the glucuronide. Gastric absorption of carbenoxolone may be necessary for the increased production of gastric mucus observed with the drug and hence is necessary for its gastric ulcer-healing activity.

Carbenoxolone, a drug used in the treatment of gastric and duodenal ulcer (Robson & Sullivan, 1968), has a mode of action which appears to involve the production of increased amounts of gastric mucus (Goodier, Horwich & Galloway, 1967). The drug labelled with ^{14}C is absorbed from the gastrointestinal tract in the rat and metabolized to conjugates of carbenoxolone and β-glycyrrhetic acid, the hydrolysis product of carbenoxolone. Most of the drug and its metabolites are excreted in the bile of rat and only negligible amounts are excreted in the urine (Iveson, Parke & Williams, 1966).

The extent and site of absorption, and the rate and route of excretion, of the drug have now been studied in patients with gastric ulcers to determine whether these parameters are similar to those observed in the rat and thus to ascertain whether the mode of action of the drug is topical or systemic.

EXPERIMENTAL

Determination of carbenoxolone

The concentration of total carbenoxolone, namely carbenoxolone and any of its known conjugates and metabolites, present in whole blood (heparinized), urine, gastric contents and bile, collected at intervals from patients with gastric ulcer receiving carbenoxolone, was determined spectrophotometrically (Coleman & Parke, 1963). The carbenoxolone sodium was taken as a single dose of 200 mg (50 mg tablets) with a little water on an empty stomach.

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Determination of carbenoxolone and its metabolites excreted in bile

Patients with T-tube drainage after cholycystectomy were given a single oral dose of 100 or 200 mg of carbenoxolone sodium on an empty stomach and the total carbenoxolone of the bile collected by drainage determined (Coleman & Parke, 1963).

The bile was also examined for metabolites by chromatography on thin-layer plates (0·25 mm) of fluorescent silica gel HF 254 (Merck) developed in the solvent system A: acetic acid–1,2-dichloroethane–n-butanol–water (4:4:1:1 by vol.). Carbenoxolone and its metabolites (see Table 1 for Rf values) were detected by their characteristic quenching of the background fluorescence when viewed under ultraviolet light (Chromatolite lamp). Glucuronide conjugates were detected by spraying the chromatograms with a mixture of 0·2% w/v naphthoresorcinol in acetone and 9% v/v aqueous phosphoric acid (5:1 by volume) and heating at 120° for 10 min; glucuronides appeared as violet spots on a buff background. The C-30 ester glucuronide of carbenoxolone and the 3-*O*-sulphate ester of β-glycyrrhetic acid were identified by comparison with authentic synthetic materials (Iveson & others, 1966; Iveson & Parke, 1970).

Determination of carbenoxolone and β-glycyrrhetic acid in mixtures

Carbenoxolone and β-glycyrrhetic acid were quantitatively determined in mixtures by a modification of the method of Coleman & Parke (1963). Solutions of mixtures in chloroform were chromatographed on thin-layer plates (0·25 mm) of silica gel HF245 in solvent system B: n-butanol–aqueous ammonia solution (s.g. 0·88) (5:1 by volume) (see Table 1 for Rf values). The bands corresponding to carbenoxolone

Table 1. *Rf values of carbenoxolone and its metabolites.* Compounds were chromatographed on thin-layer plates (0·25 mm) of silica gel HF 254 developed in the solvent system A: acetic acid–1,2-dichloroethane–n-butanol–water (4:4:1:1 by vol); or solvent system B: n-butanol–aqueous ammonia (s.g. 0·88) (5:1 by vol)

Compound	Rf value in Solvent A	Rf value in Solvent B
Carbenoxolone	0·95	0·15
Carbenoxolone-30-glucuronide	0·85	—
β-Glycyrrhetic acid	0·95	0·40
β-Glycyrrhetic acid-3-sulphate	0·75	—
β-Glycyrrhetic acid-30-glucuronide	0·80	—
β-Glycyrrhetic acid diglucuronide	0·60	—

and β-glycyrrhetic acid were located by reference to authentic materials under ultraviolet light (Chromatolite lamp) and were separately excised, transferred to tapered centrifuge tubes and the triterpenoids quantitatively eluted from the silica gel by shaking with 3·0 ml of ethanol. The silica gel was deposited by centrifugation and the extinction of the solutions of carbenoxolone and β-glycyrrhetic acid was determined at 248 nm (Coleman & Parke, 1963).

Recoveries of known amounts of carbenoxolone and β-glycyrrhetic acid (5–50 μg) separately, and in various mixtures of both, were all 100 ± 5% of the added amounts.

Effect of gastric pH on absorption of carbenoxolone

A single oral dose of carbenoxolone sodium (150 mg) was administered together with 120 ml 0·05M sodium citrate buffer, pH 8·5, to three normal subjects. Samples (5 ml) of gastric contents were aspirated at intervals and samples of blood (heparinized) were taken simultaneously. The total carbenoxolone content of the blood was determined as previously described.

The pH values of the samples of gastric aspirates were measured, and the concentration of carbenoxolone and its hydrolysis product, β-glycyrrhetic acid, were quantitatively determined by the method previously described for mixtures of these two terpenoids. Gastric aspirates (3 ml) were adjusted to pH <1 with 2N HCl and the total carbenoxolone plus β-glycyrrhetic acid was extracted with 2 × 2·5 ml portions of chloroform. The bulked chloroform extracts from each sample of gastric aspirate were dried over anhydrous sodium sulphate, filtered and concentrated to 1·0 ml. Samples of 0·2–1·0 ml of the concentrated chloroform extract were applied to thin-layer plates of silica gel HF 254 together with reference spots (50 μg) of authentic carbenoxolone and β-glycyrrhetic acid. The two compounds were then separated and quantitatively determined.

RESULTS

Concentration of carbenoxolone in blood, urine and gastric contents

The concentration of total carbenoxolone present in the blood at various time intervals after oral dosage of patients with carbenoxolone sodium is summarized in Fig. 1. The characteristic pattern of blood concentration shows an initial maximum

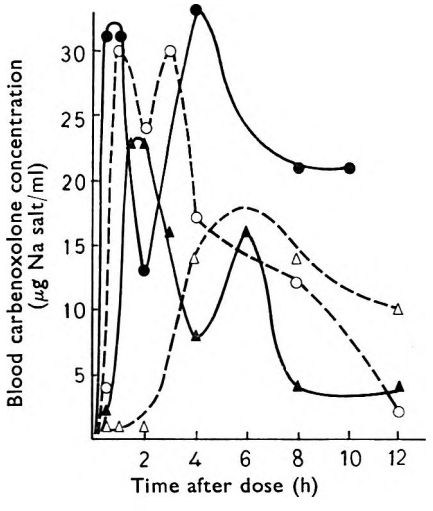

FIG. 1. Concentration of carbenoxolone sodium in whole blood of patients I (male) (●—●), II (female) (○ - - - ○), III (female) (▲—▲) and IV (male) (△ - - - △) at different periods of time after oral administration of 200 mg carbenoxolone sodium.

at 1–2 h and a second maximum occurring at 3–6 h after dosage. Furthermore, from some of the blood concentration curves it could be inferred that a progression of decreasing concentration maxima may even occur (see patients I and II in Fig. 1) In two patients (see patient IV in Fig. 1) there was a delay in attaining significant

blood concentrations and these never reached those exhibited by the other patients. This delayed absorption may have been due to the premature taking of food. In 10 patients the first maximum had a mean value of 24 μg carbenoxolone sodium/ml of blood within the range of 13–35 μg/ml; the corresponding values for the second maximum were 20 μg/ml (3–32 μg/ml) and at 12 h after dosage were 4 μg/ml ($<$1–10 μg/ml). However, there were cases (e.g. patient I in Fig. 1) where the second maximum was greater than the first. The mean value of 24 μg/ml for the first maximum, for a blood volume of 5 litres, corresponds to a total of 120 mg carbenoxolone sodium, equivalent to 60% of the dose. The maximum value of 35 μg/ml similarly corresponds to 88%, of the dose.

The concentration of carbenoxolone and its metabolites in the urine samples were always lower than the corresponding concentrations in the blood (see Table 2). In patients with high blood concentrations, the maximum urine concentrations of drug and metabolites occurred immediately after the blood maxima. In the two patients not showing high blood maxima urine concentrations of the drug were negligible. Assuming an average output of 50 ml of urine per hour, the total carbenoxolone excreted in 12 h would be equivalent to a maximum of 4 mg of carbenoxolone sodium, or 2% of the dose administered. The chemical identity of the material excreted in the urine was not determined.

The highest concentration of carbenoxolone in solution in the gastric contents was 130 μg/ml at 0·5 h after dosage in patient IV who exhibited delayed absorption of the drug (see Fig. 1). In the other patients the maximum concentration of drug was always $<$50 μg/ml and progressively declined with time, although trace amounts of the drug ($<$10 μg/ml) were still present 10 h after dosage (see Table 2). These low concentrations of carbenoxolone in solution were due largely to its insolubility at the acid pH of the stomach contents. As the total volume of the gastric contents, and other factors such as the rate of gastric emptying, the extent of duodenal reflux, and the rate of disintegration of the carbenoxolone tablets, were unknown, concentration of the drug in the gastric contents was not of great significance.

Table 2. *Concentration of carbenoxolone in the blood, urine and gastric contents of patient I*

Time after dosage (200 mg Na salt) (h)	Concentration of carbenoxolone (μg Na_2 salt/ml) in Blood	Urine	Gastric contents
0·5	31	3	34
1	31	5	9
2	13	3	5
4	33	4	$<$2
8	21	10	7
10	21	7	9

Excretion of carbenoxolone and its metabolites in bile

The concentration of carbenoxolone and its metabolites in bile was determined in four cholycystectomized patients who had subsequently received carbenoxolone sodium. Two of these patients did not show signs of jaundice and the total amount of carbenoxolone excreted in their bile in 48 h was 20·6 and 19·7% of the dose. In

both, the highest concentrations (79 and 125 μg/ml) occurred 6–12 h after dosage. Details of the first of these two cases are given in Table 3. The other two patients did show signs of jaundice and the total carbenoxolone excreted in the bile in 48 h was 14·8 and 4·8% of the dose; the highest concentrations were 62 μg/ml in the 18–24 hour period in the first of these two subjects and 33 μg/ml in the 6–12 h period in the second subject. The lower rates observed in the jaundiced patients may be indicative of impaired hepatic conjugation and excretion, or could simply be due to differences in the extents of drainage.

Table 3. *Excretion of carbenoxolone and its metabolites in the bile**

Time after dosage (200 mg Na salt) (h)	Volume of bile collected (ml)	Carbenoxolone		
		Concentration (μg Na_2 salt/ml)	Total amount (mg Na_2 salt)	% Dose
0–6	136	38	5·1	2·6
6–12	150	79	11·9	5·9
12–18	135	73	10·1	5·1
18–24	132	46	6·1	3·1
24–30	144	26	3·8	1·9
30–36	124	16	2·2	1·1
36–42	114	13	1·4	0·7
42–48	96	3	0·3	0·2
				20·6

* This patient was not jaundiced

With T-tube drainage of the biliary tree not all of the bile secreted is collected, and an unknown and variable amount by-passes the drainage tube and enters the duodenum. The total amount of the drug excreted in the bile of the non-jaundiced patients, about 20%, of the dose, is therefore a fraction of the total biliary excretion. The total volumes of bile collected in 48 h from these two patients were 1·03 and 0·82 litres. Assuming a normal average excretion of bile of 1·2 litres/day it is likely that only 30 to 40% of the total bile secreted was collected, so that the total amount of carbenoxolone excreted by this route, in 48 h, could be 50–70% of the dose.

Chromatography of the bile on thin-layer plates of silica gel revealed one major metabolite with the Rf value and characteristic naphthoresorcinol colour reaction of carbenoxolone-30-glucuronide, together with possible traces of other metabolites, particularly β-glycyrrhetic acid-3-sulphate.

Effect of pH of gastric contents on absorption of carbenoxolone

The simultaneous oral administration of an alkaline buffer solution of sodium citrate with carbenoxolone sodium had the anticipated effect of neutralizing the normal gastric acidity, raising the pH of the gastric contents and therefore increasing the proportion of the drug present in the ionized form. In one subject VI (see Table 4), the pH of the stomach contents, initially 7·5–8, fell steadily over 2 h to 3·1. Only traces of carbenoxolone were found in the blood during this period. With the second subject, V (see Table 4), the pH of the stomach contents fell during the first hour from the initial value of 7·5–8 to 1·9 and then to 1·3 in the second hour. The blood concentrations of carbenoxolone during the first hour were negligible but

Table 4. *Effect of pH of gastric contents on absorption of carbenoxolone.* The sodium salt (150 mg) was administered orally simultaneously with 120 ml 0·05M sodium citrate buffer pH 8·5

	Subject V (male)				Subject VI (male)			
		Gastric contents				Gastric contents		
Time after dosage (h)	Blood concn of carbenoxolone (μg/ml)	pH	Carbenoxolone concn (μg/ml)	β-Glycyrrhetic acid concn (μg/ml)	Blood concn of carbenoxolone (μg/ml)	pH	Carbenoxolone concn (μg/ml)	β-Glycyrrhetic acid concn (μg/ml)
0	<1	7·4	260	13	<1	7·5	75	5
0·25	—	7·9	195	7	—	6·9	70	5
0·5	<1	5·4	65	3	<1	8·2	82	7
0·75	—	2·2	52	3	—	6·3	95	6
1·0	<1	1·9	90	3	<1	6·0	72	5
1·25	—	1·7	25	1	—	5·9	38	3
1·5	9	1·5	25	1	1	5·8	24	1
1·75	—	1·5	39	1	—	4·5	50	2
2·0	12	1·3	25	1	1	3·1	18	<1

during the second hour reached 12 μg/ml. The third subject was similar to the second, the pH falling from 7·3 to 2·0 after 1 h and to 1·5 after 2 h; blood concentrations of carbenoxolone were <1 μg/ml during the first hour but reached 10 μg/ml at the end of the second hour. Appreciable concentrations of carbenoxolone therefore do not appear in the blood until the pH of the stomach contents falls below 2.

Determination of the content of carbenoxolone and β-glycyrrhetic acid present in the stomach contents shows that at pH 7·5–8 the concentrations of carbenoxolone in solution (75 and 260 μg/ml) are higher than those observed at lower pH values or in patients not receiving the alkaline buffer (see Table 2). The concentrations of the hydrolysis product, β-glycyrrhetic acid, are some 5% of the carbenoxolone concentration and do not increase with time. Carbenoxolone sodium in solution normally contains some 5% of β-glycyrrhetic acid, so that there is no evidence of further hydrolysis of carbenoxolone to β-glycyrrhetic acid after incubation in the stomach with gastric contents, either at acid or neutral pH values.

DISCUSSION

Many acidic drugs are absorbed from the stomach, the rate of absorption being dependent primarily on the dissociation constant of the drug and the lipid-solubility of its unionized molecule (Schanker, Shore & others, 1957; Schanker, 1964; Kakemi, Arita & others, 1967). Carbenoxolone is a weak acid (pK′, 6·7; pK″, 7·1) that is highly lipid soluble in its non-ionized state. The distribution coefficients for carbenoxolone at 24° between chloroform and aqueous buffers are 2 at pH 7·4 and >100 at pH 1·0, and between n-octanol and aqueous buffers are 9 at pH 7·4 and >100 at pH 1·0 (Lindup, personal communication). It would therefore be reasonable to expect carbenoxolone to be readily absorbed from the stomach, where normally it would not be significantly ionized (0·0002% at pH 1·0). However as β-glycyrrhetic acid, the parent substance, was found to be largely excreted unchanged in the faeces when administered orally to man, and hence was assumed to be largely unabsorbed (Carlat, Margraf & others, 1959), it was thought that carbenoxolone also would be largely unabsorbed and that its action in the healing of gastric ulcers would be essentially topical. These views were questioned when studies using isotopically-labelled β-glycyrrhetic acid and carbenoxolone showed both compounds to be readily

absorbed in the rat after oral administration (Parke, Pollock & Williams, 1963; Iveson, Parke & Williams, 1966).

The present results show that carbenoxolone is extensively and rapidly absorbed in man after oral administration and suggest that the principal site of absorption is the stomach. The rapid attainment of high blood levels, an average of 24 μg/ml equivalent to 60% of the dose present in the blood within 1 h of dosage, suggests that most of the drug is absorbed even before gastric emptying occurs. Furthermore, where the stomach contents were buffered to pH 7·5 (85% of carbenoxolone ionized), the normal rapid absorption of the drug, as indicated by high blood concentrations, did not occur. As the gastric acidity was restored and the stomach contents attained pH <2 ($<0{\cdot}002\%$ of carbenoxolone ionized) the drug appeared in the blood. Thus it is when the drug is largely non-ionized that absorption occurs most readily, and the major part from the stomach.

With aspirin, a typical acidic drug, absorption in dog and man is increased at low pH values because of decreased ionization of the drug, and is also increased at high pH values by virtue of the increased solubility of the drug (Truitt & Morgan, 1964). Further, the pH-dependency of the absorption of barbiturates from the stomach of the rat is marked only with those compounds, such as hexobarbitone, which, in their molecular form, have a high lipid solubility and hence are rapidly absorbed (Kakemi & others, 1967). Carbenoxolone is not readily soluble in aqueous media, even at neutral pH values, and in the unionized state is highly lipid-soluble, so that its absorption would be expected to be markedly affected by the intra-gastric pH, as has been observed. Moreover, the high degree of binding of carbenoxolone to plasma protein would have an effect on the equilibrium of the drug between the gastric contents and the blood that would result in an acceleration of the absorption process.

Analysis of the stomach contents showed that over 2 h no appreciable hydrolysis of carbenoxolone to β-glycyrrhetic acid occurs, even at pH 8, which favours the hydrolysis of the drug. Moreover, it was present in the blood unchanged and mostly excreted in the bile as the glucuronide. Thus, in contrast to the rat in which it is largely hydrolysed to β-glycyrrhetic acid before absorption (Iveson & others, 1966), in man carbenoxolone is absorbed largely unchanged.

The high blood concentrations obtained in these absorption studies suggests that most of the drug is in the circulating blood, so that although carbenoxolone is readily absorbed it does not appear to equilibrate readily with the remaining tissues of the body. This could indicate a high degree of binding of the drug to the plasma proteins. Preliminary experiments *in vitro* with human blood have confirmed this; at a plasma concentration of 25 μg/ml all the carbenoxolone was bound to plasma proteins and no unbound drug could be detected. Sulphachloropyridazine, a drug highly bound to plasma proteins, at an oral dose of 1 g gave a maximum plasma concentration in man of 100 μg/ml, which is equivalent to some 40% of the dose being present in the plasma (Newbould & Kilpatrick, 1960). With carbenoxolone, the peak blood concentrations were equivalent to nearly twice this, namely 60–70% of the oral dose present in the blood.

In man, as in the rat, only traces of carbenoxolone and its metabolites were found in the urine, and the total amount of the drug excreted by this route is unlikely to exceed 5% of the dose. The principal route of excretion of carbenoxolone in man,

as in rat, has been shown to be via the bile, but whereas in rat bile the principal metabolites are the glucuronide and sulphate conjugates of β-glycyrrhetic acid, in man the major biliary excretion product is carbenoxolone glucuronide. It would thus appear that the ester linkage of carbenoxolone is stable in man, not only in the gastro-intestinal tract before absorption, the site of its hydrolysis to β-glycyrrhetic acid in the rat, but also during the transport of the drug in the blood and its passage through the liver into the bile. The high excretion of carbenoxolone and its metabolites in the bile is more likely to be due to their high molecular weight (carbenoxolone mol. wt, 571) (see Williams, Millburn & Smith, 1965) than to the high degree of their binding to plasma proteins, since the protein-bound sulphachloropyridazine (mol. wt = 285) is readily excreted in the urine (Newbould & Kilpatrick, 1960), and indomethacin (mol. wt = 358), also highly protein-bound, is excreted approximately equally in both urine and bile (Hucker, Zacchei & others, 1966).

Drugs that are excreted in the bile as conjugates may undergo hydrolysis in the small intestine followed by reabsorption of the drug which is then excreted again in the bile, thus giving rise to an enterohepatic circulation of the drug (Williams & others, 1965). It is therefore possible that carbenoxolone, which is almost exclusively excreted in the bile, may also undergo enterohepatic circulation. Because of the rapid absorption of the drug and its confinement to the blood plasma compartment any reabsorption of drug following the secretion of bile into the intestine should be reflected in an immediate rise in the blood levels of carbenoxolone. Furthermore, the periodic emptying of the gall-bladder would be expected to give rise to a parallel periodicity in the blood concentration. Such a periodicity has been observed, resulting in maximum blood concentrations of the drug at approximately one and four h after dosage. This is strongly indicative of the enterohepatic circulation of carbenoxolone. An alternative explanation of this periodicity is that it could be attributed to the sequential absorption of the drug, first from the stomach and then of the remainder from the small intestine after gastric emptying. This is unlikely, since the peak blood concentrations are each equivalent to approximately 60% of the dose and in some cases the second peak was even greater than the first one.

It is suggested that the ulcer-healing action of carbenoxolone is the result of an increase in gastric mucus (Goodier & others, 1967). This could be due to increased secretion or to an increased rate of biosynthesis of glycoprotein, but either mechanism would probably require the passage of the carbenoxolone into the cells of the gastric mucosa, which is likewise the first requirement for the absorption of the drug. Absorption of carbenoxolone from the stomach may therefore be a prerequisite for its gastric-ulcer healing activity.

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The effect of directly and indirectly acting sympathomimetic amines on bronchospasm in the guinea-pig during CO_2 inhalation

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The bronchodilator effects of nine sympathomimetic amines were tested by reduction of histamine-induced bronchospasm in guinea-pigs. Their order of potency was: isoprenaline>adrenaline>noradrenaline > orciprenaline > ethylnoradrenaline > phenylephrine > amphetamine=tyramine>ephedrine. In guinea-pigs ventilated with 10% CO_2 in air, there was a marked decrease in the bronchoconstrictor activity of histamine. Under these conditions, isoprenaline, adrenaline, noradrenaline and orciprenaline were as effective as bronchodilators as they had been in guinea-pigs ventilated with air, ethylnoradrenaline was less effective, phenylephrine, amphetamine and tyramine were completely inactive, and ephedrine potentiated the bronchoconstrictor action of histamine. After returning the guinea-pigs to ventilation with air, the bronchoconstrictor activity of histamine and the bronchodilator effects of the sympathomimetic amines were restored. The cardiovascular effects of histamine and of all the sympathomimetic amines used were diminished during ventilation with 10% CO_2 in air and were restored after ventilation with air was resumed.

The sympathomimetic amines commonly employed as bronchodilators in asthma have been investigated by Atkinson & Rand (1968) for their alleged acute cardiac toxicity. These authors found that, in cats, during infusions of either adrenaline, isoprenaline or orciprenaline, there was suppression of the cardiovascular effects involving β-receptors of a single injection of any of these drugs.

In further experiments it was found that in cats ventilated with 10% CO_2 in air, the cardiovascular effects of adrenaline, isoprenaline or orciprenaline involving β-receptors were reduced or abolished (Atkinson and Rand, unpublished). It was therefore decided to examine the effects of sympathomimetic amines during CO_2 inhalation on histamine-induced bronchospasm, to determine whether suppression of β-receptor activity also occurred in bronchial smooth muscle.

EXPERIMENTAL

Methods

Guinea-pigs of either sex, 220–580 g, were anaesthetized by intraperitoneal injection of 4 ml/kg of a solution containing 25% urethane and 1% chloralose in saline. Blood pressure was monitored from the left common carotid with a Statham transducer (type P23AA) coupled with a strain gauge coupler (Offner No. 9872). Needle electrodes were inserted under the skin and connected to a cardiotachometer coupler (Offner No. 9857). The trachea was cannulated and artificial ventilation was applied with a constant volume Palmer respirator. A side-arm on the tracheal cannula was

connected with a Statham transducer to measure changes in intratracheal pressure via a strain gauge coupler. The recordings of heart rate, blood pressure and intratracheal pressure were displayed on an Offner Dynograph (Type R).

Arterial blood samples (0·15 ml) were withdrawn from the blood pressure cannula into a length of nylon tubing and blood pH, pCO_2 and pO_2 were measured at 37° with an IL ultramicro pH-gas Analyser (Model 113-S1). Heparin was routinely administered when the blood pressure cannula was inserted, and a small volume of dilute heparin was introduced into the arterial cannula after each blood sample was taken.

During the initial period of each experiment, blood pH was measured and the stroke volume of the respiratory pump was adjusted to stabilize the blood pH as close to 7·4 as possible. Control responses to histamine injections were then elicited at 6 min intervals until the transient increases in intratracheal pressure produced by histamine became constant. The effect of histamine in increasing intratracheal pressure was taken to represent bronchoconstriction. The bronchodilator drugs were injected 20 s before the histamine injection and the effect on bronchoconstriction was observed during the control period, during a period of ventilation with 10% CO_2 in air, and for a period after returning to ventilation with air. Blood pH, pCO_2 and pO_2 were measured during all three periods.

The dose of histamine was adjusted during the period of ventilation with 10% CO_2 in air, and after resuming ventilation with air, so that the bronchoconstrictor response matched that obtained in the control period.

Histamine bronchoconstriction and its modification by the various sympathomimetics was expressed as follows. The basal intratracheal pressure produced by artificial ventilation immediately before an injection was taken as unity, and the increase above this pressure produced by the injection of histamine was expressed as a multiple of the basal pressure. The effect of a bronchodilator sympathomimetic drug was expressed as the percentage change in this index of the bronchoconstrictor effect of histamine.

Drugs used were: histamine acid phosphate (British Drug Houses Ltd.), (±)-isoprenaline hydrochloride (Isuprel, Winthrop Laboratories), (−)-adrenaline tartrate (Adrenate, Burroughs Wellcome and Co. Ltd.), (−)-noradrenaline bitartrate (Levophed, Winthrop Laboratories), orciprenaline sulphate (Alupent, Boehringer Ingleheim Pty. Ltd.), (±)-ethylnoradrenaline hydrochloride (Bronkephrine, Winthrop Laboratories), (−)-phenylephrine hydrochloride (Neosynephrine, Winthrop Laboratories), tyramine hydrochloride (Koch-Light Laboratories Ltd.), (±)-amphetamine sulphate (L. Light and Co. Ltd.), ephedrine hydrochloride (D.H.A. Laboratories Pty. Ltd.).

RESULTS

Effect of ventilation with 10% CO_2 on histamine bronchospasm

The mean dose of histamine required to produce a bronchospasm approximately 1·5 times the basal intratracheal pressure was 4·8 μg/kg in 56 guinea-pigs. When the guinea-pigs were ventilated with 10% CO_2 in air the response to histamine started to decrease immediately. The sensitivity to histamine usually stabilized within 30 min, at which time almost five times the dose of histamine was required to produce an

approximately equivalent bronchoconstrictor effect to that obtained during ventilation with air; the mean dose of histamine required to match approximately the degree of bronchospasm was increased to 19·7 μg/kg. The sensitivity to histamine usually returned to the initial control level within an hour of restoring the animals to ventilation with air. The data on the mean doses of histamine and mean degrees of bronchospasm produced during the three phases of the experiments are summarized in Table 1.

Effect of ventilation with 10% CO_2 in air on blood pH and pCO_2

In the control period during ventilation with air, a mean blood pH of 7·41 was obtained by adjusting the stroke of the respiration pump and the mean pCO_2 figure was 28·3 mm Hg. The pCO_2 is relatively low compared with figures for human arterial blood: the reason for this is not known. Ventilation with 10% CO_2 in air for 30 min resulted in an increase of the mean arterial blood pCO_2 to more than 95 mm Hg; and the mean pH was reduced to 6·81. When the guinea-pigs were returned to ventilation with air, these values tended to return to control levels. The data are summarized in Table 1.

Table 1. *Effects of ventilation with 10% CO_2 in air on histamine bronchospasm and pH and pCO_2 in guinea-pigs*

Parameter mean ± s.e.	Air	10% CO_2 in air for 30 min	Resumption of air for 60 min
Histamine dose, μg/kg	4·8 ± 0·4	19·7 ± 2·3	4·5 ± 0·7
Degree of bronchospasm (increase/basal tracheal pressure)	1·53 ± 0·07	1·30 ± 0·07	1·47 ± 0·13
pH	7·41 ± 0·11	6·81 ± 0·16	7·28 ± 0·09
pCO_2..	28·31 ± 1·41	96·85 ± 3·33	32·79 ± 2·49

Effects of sympathomimetic drugs on histamine bronchospasm

Nine sympathomimetic amines were examined for their ability to reduce histamine-induced bronchoconstriction. The range of doses of each which reduced the bronchoconstrictor effect of histamine by about one-third was determined in preliminary experiments. They are arranged in rank order of potency, as indicated by the mean doses producing mean reductions of 23 to 55% in histamine bronchospasm, in Table 2.

Table 2. *Effects on histamine-induced bronchoconstriction of sympathomimetics, before, during and after ventilation with 10% CO_2 in air*

Sympathomimetic	Dose (μg/kg) mean ± s.e. (No. of experiments)	Percentage reduction of histamine-induced bronchospasm mean ± s.e. (No. of experiments)		
		Before CO_2 ventilation	During CO_2 ventilation	After CO_2 ventilation
Isoprenaline ..	0·079 ± 0·016 (8)	36·5 ± 5·4 (8)	33·2 ± 8·5 (8)	38·2 (2)
Adrenaline ..	0·104 ± 0·019 (5)	54·3 ± 2·5 (5)	46·2 ± 3·2 (5)	67·0 (2)
Noradrenaline ..	0·700 ± 0·100 (3)	44·7 ± 7·7 (3)	42·3 ± 6·7 (3)	48·5 (2)
Orciprenaline ..	1·63 ± 0·28 (6)	31·3 ± 6·3 (6)	25·4 ± 7·3 (6)	35·5 ± 7·9 (3)
Ethylnoradrenaline	4·63 ± 1·74 (3)	52·0 ± 8·5 (3)	24·8 ± 8·5 (3)	53·5 (2)
Phenylephrine ..	8·70 ± 3·84 (4)	52·2 ± 8·1 (4)	−4·3 ± 1·2 (4)	20·5 ± 0·5 (3)
Amphetamine ..	2000 ± 400 (7)	28·8 ± 3·1 (7)	−0·6 ± 2·3 (7)	34·7 ± 14·4 (3)
Tyramine ..	2100 ± 400 (6)	23·3 ± 3·9 (6)	0·6 ± 0·6 (3)	32·0 ± 5·0 (3)
Ephedrine ..	4000 ± 0 (7)	55·0 ± 9·0 (7)	−44·8 ± 13·7 (6)	46·0 ± 15·0 (3)

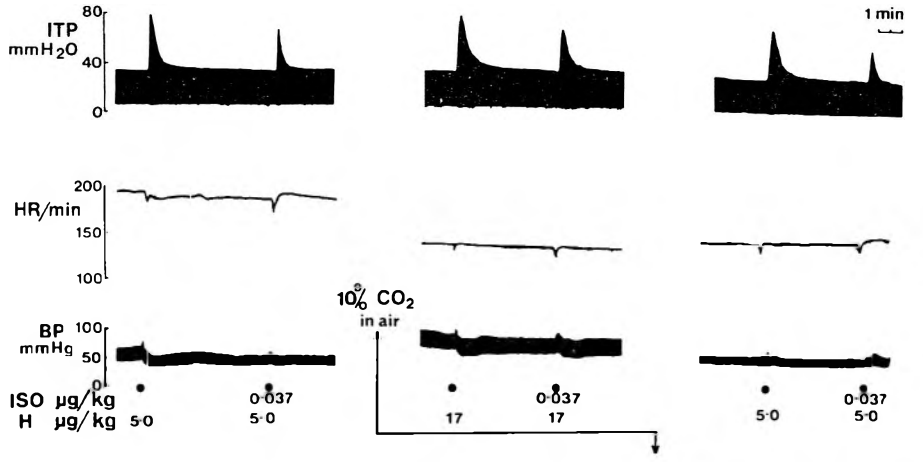

FIG. 1. Records of intratracheal pressure (ITP), heart rate (HR/min) and blood pressure (BP) in anaesthetized guinea-pig. The reduction in histamine-induced bronchospasm produced by isoprenaline (0·087 µg/kg) was unaffected by ventilation with 10% CO_2 in air.

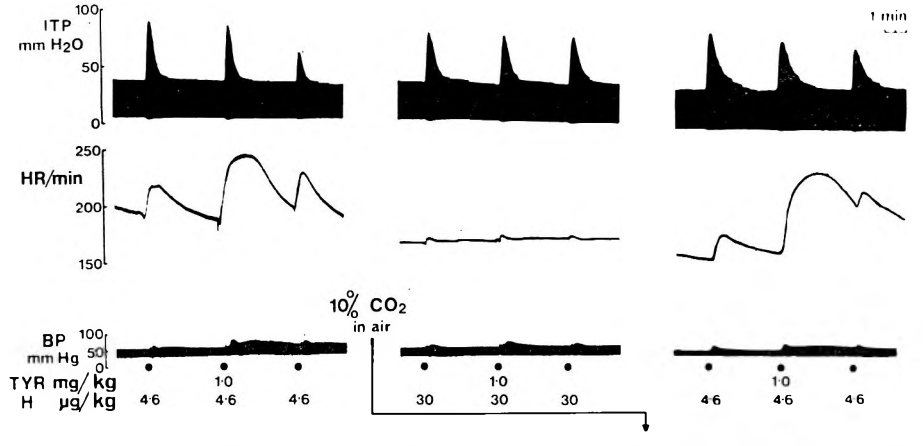

FIG. 2. Records as in Fig. 1. The reduction in histamine bronchospasm produced by tyramine (1 mg/kg) was abolished when the guinea-pig was ventilated with 10% CO_2 in air, but returned after ventilation with air was resumed.

Representative records of experiments with isoprenaline, tyramine and ephedrine are shown in Figs 1–3 (upper tracing, left-hand panel of each figure).

The rates of onset and durations of action of the various sympathomimetic amines differed. For the most potent, from isoprenaline to phenylephrine (see Table 2), the maximal action was observed in the reduction of the responses to the injection of histamine given 20 s later. Their effects were transient and generally did not last the 6 min to the next injection of histamine, although in some experiments a slight bronchodilator effect was seen with ethylnoradrenaline and phenylephrine 6 min after their injection. With amphetamine, tyramine and ephedrine, the maximal bronchodilator effect was observed 6 min after they were injected. Thus, Figs 2 and 3 show that injections of tyramine and ephedrine respectively had greater effects in

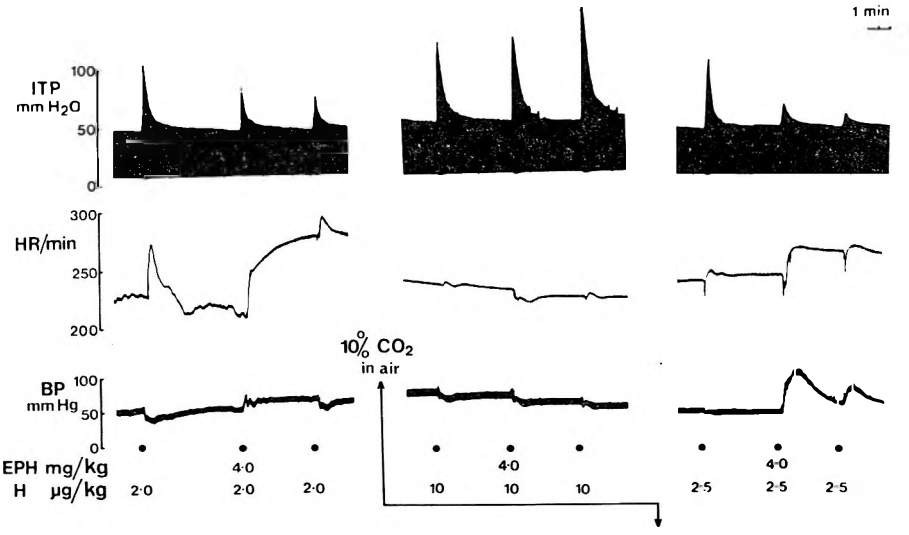

FIG. 3. Records as in Fig. 1. Ephedrine (4 mg/kg) reduced the bronchoconstrictor action of histamine during the control period, but potentiated it when the animal was ventilated with 10% CO_2 in air. The potentiation was sustained during this period. When ventilation with air was resumed, ephedrine again reduced the histamine response.

reducing the bronchoconstrictor responses to histamine given 6 min after their injection than it had on the responses to histamine given 20 s after injection of the sympathomimetic amines. The bronchodilator effects of tyramine and ephedrine lasted from 15 to 60 min. The bronchodilator effect of amphetamine was even more long lasting and never wore off entirely during the course of an experiment. Tachyphylaxis developed to the bronchodilator effect of amphetamine, a second injection being entirely without action. With the other sympathomimetic amines, reproducible responses were obtained with subsequent injections once the effect of the previous injection had passed off.

Effect of ventilation with 10% CO_2 on the bronchodilator effects of sympathomimetic amines

The reduction in histamine-induced bronchospasm produced by the four most potent sympathomimetic amines, isoprenaline, adrenaline, noradrenaline and orciprenaline was slightly but not significantly reduced when the guinea-pigs were ventilated with 10% CO_2 in air. If these sympathomimetics are considered as antagonists of the bronchoconstrictor action of histamine, then they are in fact more potent during ventilation with 10% CO_2 in air since the dose of histamine causing the effect is about five times greater than during ventilation with air. An experiment with isoprenaline is illustrated in Fig. 1 from which it can be seen that the reduction of the bronchoconstrictor effect of histamine in the second panel, during ventilation with 10% CO_2 in air, is as great as on the comparable response to a smaller dose of histamine in the first panel, during ventilation with air.

With the next most potent bronchodilator, ethylnoradrenaline, there was a significant reduction in bronchodilator effect during ventilation with 10% CO_2 in air. The mean reduction in histamine bronchospasm during ventilation with air was 52%

and the mean reduction during ventilation with 10% CO_2 in air was 24·8%, the difference between these means being significant by *t*-test with $P = 0{\cdot}05$.

The less potent sympathomimetic bronchodilators, phenylephrine, amphetamine and tyramine, lost their bronchodilator efficacy in guinea-pigs ventilated with 10% CO_2 in air, as illustrated with tyramine in Fig. 2. Because of the tachyphylaxis which developed after the first injection of amphetamine, a separate series of experiments was performed in which the effect of amphetamine was observed in guinea-pigs ventilated with 10% CO_2 in air without previous administration of a sympathomimetic amine: amphetamine did not reduce the bronchoconstrictor effect of histamine in these experiments.

Ephedrine, the least potent bronchodilator of the sympathomimetic amines tested in guinea-pigs ventilated with air during the control period, potentiated the bronchoconstrictor action of histamine when it was given to guinea-pigs ventilated with 10% CO_2 in air. An experiment illustrating this finding is shown in Fig. 3. The effect of ephedrine in increasing the bronchoconstrictor response to histamine persisted for more than an hour after the injection of ephedrine.

The bronchodilator activity of all those sympathomimetic amines which were less active or inactive during ventilation with 10% CO_2 in air was restored within 1 h of resuming ventilation with air; and occurred at approximately the same time that the sensitivity to the bronchoconstrictor effect of histamine returned to the initial control level. The bronchodilator action of amphetamine was observed after resumption of ventilation with air; the injection given during the period of ventilation with 10% CO_2 was not only ineffective in producing bronchodilatation but also did not produce tachyphylaxis. Restoration of the bronchodilator action of ephedrine occurred after ventilation with air was resumed; this required about 1 h, as with phenylephrine and tyramine.

When the bronchodilator effects of ethylnoradrenaline, phenylephrine, tyramine, amphetamine and ephedrine had been reduced, abolished or reversed by ventilation with 10% CO_2 in air, the guinea-pigs still responded to isoprenaline in the usual manner. The altered response to these amines is therefore apparently not a result of β-receptor blockade.

Blood pressure and heart rate changes

The doses of histamine used generally produced a very slight fall in blood pressure and sometimes an increase in pulse pressure. The effect on the heart rate varied: occasionally, there was a slight decrease, but usually it increased by 10 to 50 beats per min. In the doses used, adrenaline, isoprenaline, orciprenaline and noradrenaline had very little or no effect on blood pressure and heart rate. Ethylnoradrenaline and phenylephrine produced a variable small rise in blood pressure and had no effect on heart rate. With tyramine, amphetamine and ephedrine there was a prolonged increase in blood pressure or pulse pressure. Tyramine, amphetamine and ephedrine occasionally produced a slight fall in heart rate preceding a sustained rise, but more usually there was only a sustained rise.

When the guinea-pigs were ventilated with 10% CO_2 in air, the blood pressure usually increased. The immediate effect on heart rate varied from a slight fall to a slight rise, and there was a gradual fall thereafter. During the period of ventilation with 10% CO_2 in air, the decreased sensitivity to the bronchoconstrictor effects of

histamine was accompanied by a decreased sensitivity to the cardiovascular effects of histamine and all of the sympathomimetic amines used. Sensitivity to the cardiovascular effects of the sympathomimetics started to return at about the same time as bronchial sensitivity after ventilation with air was resumed.

DISCUSSION

The order of potency of a series of sympathomimetics in counteracting histamine bronchospasm in the guinea-pig was found to be isoprenaline>adrenaline>noradrenaline > orciprenaline > ethylnoradrenaline > phenylephrine > tyramine= amphetamine > ephedrine. Phenylephrine was a fairly potent antagonist of histamine-induced bronchospasm although it is regarded primarily as an α-receptor agonist (Levy & Ahlquist, 1961). The dose of phenylephrine required to reduce the histamine response by approximately 50% was only twice that of ethylnoradrenaline, which has both α- and β-agonist activity (Levy, 1959).

It has been shown in this laboratory (Atkinson & Rand, unpublished work) that the cardiovascular effects of isoprenaline, adrenaline and orciprenaline involving β-receptors are suppressed during ventilation with 10% CO_2 in air in cats. The observations on heart rate and blood pressure effects of these amines in guinea-pigs in the present experiments are in agreement with these findings. However, the effects of these three amines in counteracting bronchospasm during ventilation with 10% CO_2 in air were unaffected. This supports the proposal by other workers (Lands & Brown, 1964) that β-receptors in the cardiovascular system differ from those in respiratory smooth muscle. With ethylnoradrenaline, phenylephrine, tyramine, amphetamine and ephedrine, effects involving β-receptors on both the cardiovascular system and bronchial smooth muscle were suppressed during ventilation with 10% CO_2 in air.

An explanation for the difference between isoprenaline, adrenaline, noradrenaline and orciprenaline on the one hand, and of tyramine and amphetamine on the other may lie in their different mechanisms of action, in that the former are directly acting whereas the latter are indirectly acting (Burn & Rand, 1958). However, the mechanism by which a fall in blood pH, or a rise in blood pCO_2 results in loss of action of indirectly acting sympathomimetic amines is a matter of speculation: it may be that the process of uptake of these amines into adrenergic neurons, or the process of release of noradrenaline by them, is inhibited. The main difficulty in the way of suggesting that the difference is due to different types of sympathomimetic action is that Burn & Rand (1958) found phenylephrine to be directly acting and ethylnoradrenaline is also generally regarded as directly acting. Since there are differences between species and even between tissues in the one species in the relative extents to which a given sympathomimetic may act directly and indirectly, it would be desirable to have evidence on the particular mechanism that operates in the guinea-pig bronchial smooth muscle with these two drugs.

The pK_a values of the amines tested are all above 9, so that differences in ionization at the pH produced by ventilating the guinea-pigs with 10% CO_2 in air are unlikely to account for the different effects seen.

The enhanced bronchoconstrictor response to histamine produced by ephedrine during ventilation with 10% CO_2 in air will be subjected to further examination. It

may be due to inhibition of monoamine oxidase by ephedrine (Gaddum & Kwaitkowski, 1938) with protection of histamine from degradation (Zeller, Stern & Blacksma, 1956). Allen & Rand (1969) showed that monoamine oxidase inhibitors potentiated the bronchoconstrictor action of histamine in the guinea-pig.

The findings have implications for the treatment of asthmatic bronchospasm with commonly used sympathomimetic bronchodilators. In an attack which is so prolonged and severe that blood pCO_2 rises and pH falls, then ethylnoradrenaline may be less effective and phenylephrine ineffective, even though they may previously have been effective prophylactically or in less severe attacks; it is possible that ephedrine may even worsen the bronchospasm. In such severe asthma, isoprenaline, adrenaline and orciprenaline may still be fully effective as bronchodilators. Furthermore, in such a condition, there is no evidence to suggest increased cardiac toxicity (Atkinson & Rand, unpublished observations). However, Collins, McDevitt & others (1969) found that the cardiac toxicity of isoprenaline was increased when the blood pO_2 was decreased.

Acknowledgements

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The effect of nialamide, pargyline and tranylcypromine on the removal of amphetamine by the perfused liver

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Nialamide, pargyline and tranylcypromine, three monoamine oxidase inhibiting drugs, and the microsomal enzyme inhibitor SKF 525-A significantly decreased the amount of amphetamine removed from the portal circulation of the isolated perfused liver of the cat. The enhancement of the vascular actions and toxicity of amphetamine by monoamine oxidase inhibitors could be explained in terms of these findings.

The actions of amphetamine and its congeners are potentiated by monoamine oxidase inhibitors (Brownlee & Williams, 1963; Dally, 1962; Rand & Trinker, 1968; Sjöqvist, 1965; Trinker, Fearn & others, 1967; O'Dea & Rand, 1969; Morpurgo & Theobald, 1968). However, amphetamine is not a substrate for monoamine oxidase (Blaschko, Richter & Schlossman, 1937). The explanation of the paradox is that drugs termed "monoamine oxidase inhibitors" are not specific to that enzyme, but inhibit other enzymes including liver microsomal enzymes (Brodie, Gillette & La Du, 1958). Amphetamine is a substrate for liver microsomal enzymes (Axelrod, Reichenthal & Brodie, 1954; Axelrod, 1955), and the liver microsomal enzyme inhibitor proadifen (SKF 525-A) inhibits the metabolism of amphetamine by these enzymes *in vitro* (Cooper, Axelrod & Brodie, 1954).

The actions of amphetamine in the intact cat, as well as being potentiated by monoamine oxidase inhibitors, are also potentiated by proadifen or by excluding the liver from the circulation (Rand & Trinker, 1968). These findings suggest that metabolism of amphetamine by liver microsomal enzymes is important in limiting the pharmacological activity of amphetamine. This paper is concerned with the effects of nialamide, pargyline and tranylcypromine, representative monoamine oxidase inhibiting drugs, and of proadifen on the removal of amphetamine from a portal perfusate by the cat isolated liver.

Methods

Young cats of at least 3 months old and weighing 0·9 to 1·8 kg were used; in younger animals the liver microsomal enzyme systems are incompletely developed (Fouts & Adamson, 1959; Fouts, 1962; Conney, Schneidman & others, 1965). The cats were anaesthetized with halothane, one of the few anaesthetics that neither depress nor stimulate liver microsomal enzymes (Schimassek, Kunz & Gallwitz, 1966).

A midline abdominal incision was made from the xiphisternum to the pubic symphysis and the liver was exposed by displacing loops of the intestine to the left.

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The common bile duct and hepatic artery were tied and cut between ligatures. The portal vein was cannulated and perfusion was commenced with McEwen solution at 37° using a Watson-Marlow roller pump delivering 6 ml/min; this caused an immediate and uniform blanching of the liver parenchyma. The liver was freed from surrounding tissues and the hepatic veins and inferior vena cava were cut. The excised liver was weighed, suspended in a double jacket water bath maintained at 37° and perfused until the effluent was macroscopically free of blood.

A single dose of amphetamine was injected into the portal vein cannula in a dose equivalent to 12 μg/g of liver. A sample of effluent was collected before administration of amphetamine and thereafter serial samples were collected during 2 min periods. The amphetamine content of the effluent was determined spectrophotometrically by the method of Chapman, Shenoy & Campbell (1959) using the methyl orange reaction and adsorption at 515 μm. A standard curve was constructed for each experiment. Duplicate aliquots of each test sample were assayed.

Cats were pretreated as described by Rand & Trinker (1968), monoamine oxidase inhibitors being given intraperitoneally 16 to 18 h and proadifen 1 h before removing the liver.

The drugs used were (±)-amphetamine sulphate, nialamide hydrochloride, pargyline hydrochloride, proadifen hydrochloride (SKF 525-A) and tranylcypromine sulphate. The amounts referred to in the text are in terms of these salts.

RESULTS

No amphetamine was detected in the liver perfusate of both control and pretreated cats before administration of amphetamine. Our colleague Mr. H. J. Fearn also tested the samples for catecholamine activity by bioassay using the blood pressure of the pithed rat and the de Jalon preparation of the rat uterus, and obtained negative results.

In preliminary experiments it was established that after injection of amphetamine, the concentration in the effluent fell below detectable levels within 10 min. The mean total amount of amphetamine recovered in the effluent fluid from perfused livers of control untreated animals was 34·6% of that administered. Pretreatment with any of the three monoamine oxidase inhibitors resulted in substantial and highly significant increases in the amounts of amphetamine appearing in the effluent. The means, standard errors, and significances of difference from control values are summarized in Table 1. The monoamine oxidase inhibiting drugs included a hydrazine derivative (nialamide), two non-hydrazines (pargyline and tranylcypromine), and a drug having amphetamine-like sympathomimetic activity (tranylcypromine). Pretreatment with the microsomal enzyme inhibitor SKF 525-A had

Table 1. *Recovery of amphetamine from isolated perfused cat liver* %

Treatment	No. of animals	% recovery ± s.e.	*P* value
Control	9	34·6 ± 3·9	
Nialamide (50 mg/kg)	9	64·5 ± 4·7	<0·001
Pargyline (50 mg/kg)	5	68·8 ± 3·9	<0·001
Tranylcypromine (25 mg/kg)	3	60·0 ± 3·8	<0·001
SKF 525-A (20 mg/kg)	4	62·5 ± 6·4	<0·001

a similar effect to the monoamine oxidase inhibitors in increasing the amount of amphetamine appearing in the effluent from the liver (Table 1).

DISCUSSION

Only about one-third of the amphetamine injected into the perfusion fluid appeared in the effluent from the liver. This indicates that the liver takes up amphetamine avidly. The dose of amphetamine injected into the fluid perfusing the liver (12 μg/g of liver) is equivalent to the amount of 0·09 μmol/g which was found by Axelrod (1955) to be sufficient to saturate the liver microsomal enzymes *in vitro*. When the liver microsomal enzymes were inhibited by pretreatment of donor cats with monoamine oxidase inhibitors or proadifen, about two-thirds of the dose of amphetamine appeared in the effluent. How the metabolism of amphetamine by the liver is impaired is not clear; inhibition of the enzymes is probably not involved, but there may be a change in permeability of the microsomal membrane (Brodie, 1956; Netter, 1962), or in the endoplasmic reticulum affecting the access of drugs to the microsomes (Fouts, 1965). In any event, the microsomal enzyme system as a whole is efficient in removing amphetamine from the portal circulation. In intact animals the extent of potentiation of the action of amphetamine caused by excluding the liver from the circulation was about as great as that produced by pretreatment with nialamide or proadifen; no increase in effect was produced by either nialamide or proadifen together with exclusion of the liver (Rand & Trinker, 1968). These effects are explicable in terms of the increase in the amount of amphetamine appearing in the hepatic venous effluent after its administration into the portal vein of perfused isolated livers of cats that had been pretreated with the three drugs and proadifen.

These findings offer a rational basis for the greatly enhanced pressor responses observed after sympathomimetic amines, such as amphetamine, ephedrine and methylphenidate, that are not substrates for monoamine oxidase, in animals and in man receiving monoamine oxidase inhibiting drugs.

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Selective determinations of amines and quaternary ammonium compounds as ion pairs with methyl orange by an automated method applicable to single dose analysis

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Aliphatic amines and quaternary ammonium compounds can be determined photometrically after extraction as ion pairs with methyl orange. Extraction conditions are calculated from constants for the extraction procedure and side-reactions. Methods for the selective determination of ammonium ions of different degrees of substitution are given. Quaternary ammonium ions are isolated by extraction at pH 12. The amines are extracted as ion pairs at pH 4·6 and selective determinations can be made after primary and secondary amines are masked by acetylation and primary amines by coupling with salicylic aldehyde. An automated application of the method on a Technicon AutoAnalyzer is presented. A complete analysis of a sample containing the four kinds of ammonium ions requires four separate runnings. The same basic manifold is used for all the runnings with some rearrangements for the masking procedures. The method has been tested on benzhexol, diethylpropion, dodecylamine, emepronium, *N*-hydroxyethylpromethazine, promethazine and protriptyline. Recoveries of about 100% with a relative variation of 1–2% were obtained.

Several automated methods for determination of amines and quaternary ammonium compounds based on ion pair extraction have been published (Häussler & Hajdù, 1964; Kabasakalian, Karl & Townley, 1967; Kuzel, 1968; Stevenson & Comer, 1968). The applicability of these methods is limited because of the empirical approach to extraction conditions. A theoretical approach in the method construction (Schill, 1965) gives more rapid and reliable results. The conditions are then calculated from measured values of extraction constants of the ion pairs, and from partition coefficients and dissociation constants of the amines and the anion used.

This principle was applied by Eriksson & Nyberg (1967) in an automated method on the AutoAnalyzer intended for analysis of single tablets. The organic ammonium ions were extracted by chloroform as ion pairs with methyl orange. The method gave the total ammonium content. In the present paper this method has been developed to give possibilities for selective determinations of ammonium ions of different degrees of substitution at the nitrogen.

The ion pair extraction

The ion pair extraction can be illustrated by the following reaction

$$HA^{+}_{aq} + M^{-}_{aq} = HAM_{org}$$

where HA^+ is an ammonium ion, M^- the anion of methyl orange and HAM the extracted ion pair. The equilibrium is expressed by the extraction constant, E_{HAM},

defined by the following equation

$$\frac{(HAM)_{org}}{(HA^+)_{aq} \times (M^-)_{aq}} = E_{HAM} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

The extraction can be influenced by different side reactions such as protolysis, partition of the amine as base or dimerization of the ion pair in the organic phase. It is convenient to combine the effects of all processes influencing the extraction into a conditional extraction constant, E^x_{HAM}, valid under stated conditions (e.g. pH or concentration range). The conditional extraction constant is defined by

$$\frac{C'_{HAM\ org}}{C'_A \times C'_M} = E^x_{HAM} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

$C'_{HAM\ org}$ is the total concentration of the amine present as ion pair with methyl orange in the organic phase. C'_A and C'_M represent the concentrations of all other forms of amine and methyl orange. The degree of extraction of the amine as ion pair can be expressed by the partition ratio, D_{HAM}.

$$D_{HAM} = \frac{C'_{HAM\ org}}{C'_A} = E^x_{HAM} \times C'_M \quad .. \quad .. \quad .. \quad (3)$$

The percentage degree of extraction, P, is given by

$$P = 100\left(1 + \frac{q}{D_{HAM}}\right)^{-1} \quad .. \quad .. \quad .. \quad (4)$$

where q = volume of aqueous phase/volume of organic phase. The compounds studied (1–7 in Fig. 1) represent the degrees of substitution at the nitrogen from quaternary to primary. The constants necessary for the calculation of the conditional extraction constants of their methyl orange ion pairs are given in Table 1. (The determinations of the constants will be discussed in a separate paper.)

Table 1. *Constants of extracted ammonium compounds*

Substance	Kind	Org. phase	log E_{HAM}	log $k_{2(HAM)org}$	log $K'_{HA} \times k_{d(A)}$
Emepronium ..	quatern.	$CHCl_3$	4·94	—	—
N-Hydroxyethyl-promethazine ..	quatern.	$CHCl_3$	4·74	—	—
Benzhexol	tertiary	$CHCl_3$	6·90	—	—3·06
Diethylpropion ..	tertiary	$CHCl_3$	4·24	—	—4·58
Promethazine ..	tertiary	$CHCl_3$	6·76	—	—3·00
		$C_2H_4Cl_2$	6·13	—	—3·73
Protriptyline ..	secondary	$CHCl_3$	5·27	4·48	—4·92
		$C_2H_4Cl_2$	4·68	4·71	—5·68
n-Dodecylamine ..	primary	$CHCl_3$	4·78	4·74	—4·74

$$K'_{HA} = \frac{a_{H^+} \times (A^-)}{(HA)} \qquad k_{d(A)} = \frac{(A)_{org}}{(A)_{aq}} \qquad k_{2(HAM)org} = \frac{(H_2A_2M_2)_{org}}{(HAM)_{org}}$$

The properties of methyl orange were discussed by Eriksson & Nyberg (1967). It is a sulphonic acid and an aromatic tertiary amine with $pK_a = 3{\cdot}43$ and has a solubility in water of $1{\cdot}3 \times 10^{-4}$ mol/litre. The limiting solubility of its sodium salt in 0·1M NaCl is about $10^{-2{\cdot}8}$ mol/litre. Methyl orange is not extracted from an aqueous phase by chloroform or ethylene dichloride. The degree of extraction of

the amine as ion pair will depend on E^x_{HAM} and C'_M according to equation (3). The value of the conditional constant, E^x_{HAM}, will change with pH due to protolysis and partition of the amine and protolysis of methyl orange. Furthermore, the concentration of methyl orange in the aqueous phase, C'_M, cannot exceed $10^{-2.8}$ because of its limited solubility. Calculations based on these assumptions are summarized in Fig. 1 which gives the relations between partition ratio, D_{HAM}, and pH for the compounds studied. The dimerization of primary and secondary amine ion pairs has not been considered. It is, however, a favourable effect giving a slight increase of the partition ratio in the actual concentration range.

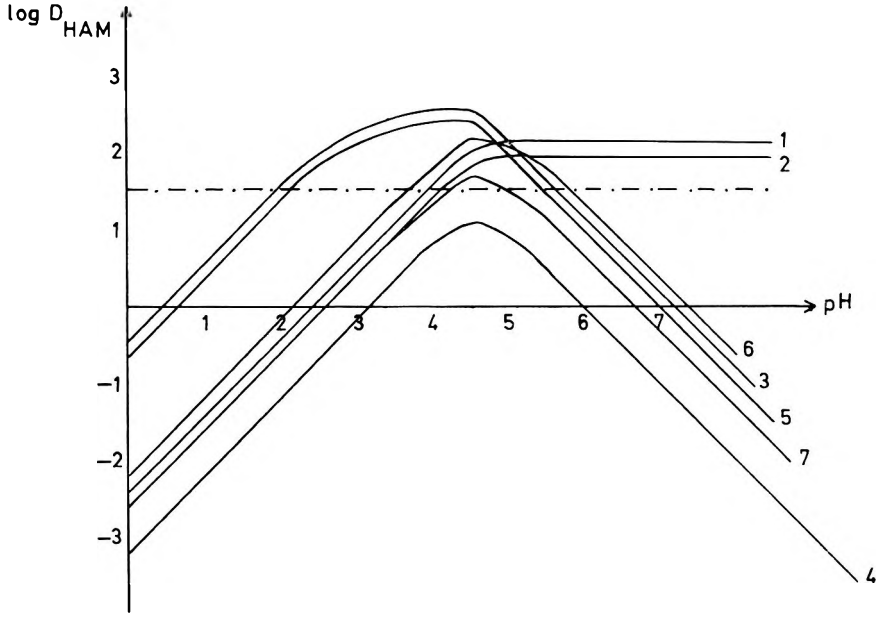

FIG. 1. Relations between maximal partition ratio and pH at the extraction of methyl orange ion pairs. 1. Emepronium. 2. *N*-Hydroxyethylpromethazine. 3. Benzhexol. 4. Diethylpropion. 5. Promethazine. 6. Protriptyline. 7. Dodecylamine. Side-reactions: protolysis and partition of the amines, protolysis and precipitation of methyl orange. Maximum concentration of methyl orange $10^{-2.8}$ M.

An extraction of more than 99% is obtained according to equation (4) if $q/D_{HAM} \leq 0{\cdot}01$. Since a volume ratio, q, of 1/3·5 is used in the automated extraction method, a partition ratio of 30 or more (log $D_{HAM} \geq 1{\cdot}5$) is then necessary for an almost complete extraction. This limit is represented by the broken line in Fig. 1.

From Fig. 1 it follows that an appropriate extraction of all the compounds is obtained at pH 4·5. With increasing pH the partition ratio of the ion pairs of the amines decreases rapidly while the quaternary ammonium ion pairs are unaffected. Thus, an extraction at pH 12 will only give the quaternary ammonium ions.

Masking reactions

Primary and secondary amines can be excluded from ion pair extraction by masking procedures giving aprotic derivatives of the amines.

Acetylation. The primary and secondary amines were acetylated with acetic anhydride to aprotic amides in a two-phase system of chloroform and an aqueous

citrate buffer with pH 4·6. The anhydride was dissolved in the chloroform in a molar concentration of 5×10^{-3} while the amine $(0{\cdot}4\text{–}1{\cdot}0) \times 10^{-4}$ was added to the aqueous phase. The phase volume ratio, chloroform : aqueous phase, was 2. The amines used in this investigation were quantitatively acetylated within 10 min at room temperature with continuous shaking of the reaction mixture.

Azomethine formation. The primary amine was coupled with salicylic aldehyde to an azomethine with only weakly basic properties. The reaction was in ethylene dichloride containing 10^{-1} mol salicylic aldehyde/litre and an aqueous borate buffer of pH 9·0 and a salicylic aldehyde concentration of 2×10^{-2}M. The phase volume ratio was 1. The amine $(0{\cdot}7\text{–}2{\cdot}0) \times 10^{-5}$M, was added to the aqueous phase and the reactants shaken at room temperature for 15 min to complete the reaction. Ethylene dichloride (b.p. 84°) was used as the organic phase, since it will admit a higher reaction temperature which might be necessary with less reactive primary amines.

Determination procedure

The reactions discussed can be combined to automated procedures for the selective determination of different kinds of amines as ion pairs with methyl orange. The principles are summarized in Table 2. A complete analysis of a mixture of

Table 2. *Principles for selective determinations of ammonium compounds*

Running	Circuit 1		Circuit 2	
	Reaction	Extraction of amines as bases	pH of aqueous phase	Determination as ion pairs
A	—	—	12·0	Quaternary ammonium ions
B	—	All	4·6	Total amine content
C	Acetylation of secondary and primary amines	Tertiary	4·6	Tertiary amines
D	Coupling of primary amines with salicylic aldehyde	Tertiary and secondary amines	4·6	Tertiary and secondary amines

four ammonium ions of different degrees of substitution requires four separate runnings. The scheme contains two circuits, one for separation and one for determination. In the determination step, the ammonium ions are transformed to methyl orange ion pairs in the organic phase which are measured photometrically at 420 nm.

Quaternary ammonium ions (running A) only require one circuit which gives both separation and determination since all amines are excluded by the use of an aqueous phase with pH 12. At this pH the amines are extracted as bases and only quaternary ammonium ions give methyl orange ion pairs. The total amine determinatio (running B) begins with an extraction of the amines as bases. The organic phase containing the amines is then treated with an aqueous solution of methyl orange with pH 4·6, which transforms the bases to methyl orange ion pairs.

In running C primary and secondary amines are acetylated and the alkalinized aqueous phase is then extracted with chloroform. In the organic phase only tertiary amines have protolytic properties giving ion pairs by treatment with methyl orange as in running B.

In running D primary amines are coupled with salicylic aldehyde. The alkalinized aqueous phase is then treated with ethylene dichloride which extracts the azomethine as well as secondary and tertiary amines as bases. Quaternary ammonium ions can also to some extent be extracted as ion pairs with salicylic aldehyde. They can be eliminated by a second treatment with an alkaline aqueous phase. The organic phase is then treated with methyl orange at which secondary and tertiary amines are transformed to ion pairs.

Reagents

NaM, stock solution (3×10^{-3}M): 0·1000 g of purified methyl orange (acid form) is dissolved in equivalent amount of NaOH, 0·1N and water to 100·00 ml. *Purified methyl orange* is prepared from a solution of the sodium salt in water by addition of HCl to pH 1, at which the acid precipitates (Eriksson & Nyberg, 1967).

Sodium borate: 0·2M borate buffer, pH 9·0, prepared from boric acid and NaOH.

Sodium citrate: 0·1M citrate buffer, pH 4·6, prepared from disodiumhydrogen citrate and NaOH. *Sodium phosphate*: 0·1M phosphate buffer, pH 12·0, prepared from trisodium phosphate and HCl. *NaM, pH* 12: a mixture of two volumes of NaM, stock solution, one volume of sodium phosphate and one volume of water. *NaM, pH* 4·6: a mixture of equal volumes of NaM, stock solution, and sodium citrate.

Acetic anhydride chloroform (Ac_2O–$CHCl_3$): 5×10^{-2}M solution of acetic anhydride in water-saturated chloroform. Freshly prepared. *SA–$C_2H_4Cl_2$*: 0·1M solution of salicylic aldehyde in water-saturated ethylene dichloride. Freshly prepared. *SA–H_2O*: saturated solution of salicylic aldehyde in water. Freshly prepared. *Brij:* 20 drops of Brij 35 (10% in water) in 1000 ml of water. All substances should be of analytical or equivalent grade.

Samples. Aqueous solutions of salts of the ammonium compounds listed in Fig. 1. In the present investigation the samples were diluted so that in each running the sum concentration of the ammonium compounds to be determined was 7×10^{-5}M.

Standards. Water solutions of a salt of an ammonium compound not being masked in the actual running. Concentrations: $3{\cdot}5 \times 10^{-5}$, $7{\cdot}0 \times 10^{-5}$ and $10{\cdot}5 \times 10^{-5}$.

Methodology

The flow schemes for a complete analysis comprising four separate runnings are given in Fig. 2. The parts of the common manifold used in each running are indicated by letters and arrows. The lettering system is the same as in Table 2. The samples are segmented with organic phase. The extractions take place in double mixing coils. Between the circuits and before measurement, desegmentation comes about in phase separators (BO in the flow scheme) at which the lighter aqueous phase goes to waste. The acetylation of primary and secondary amines takes place in a

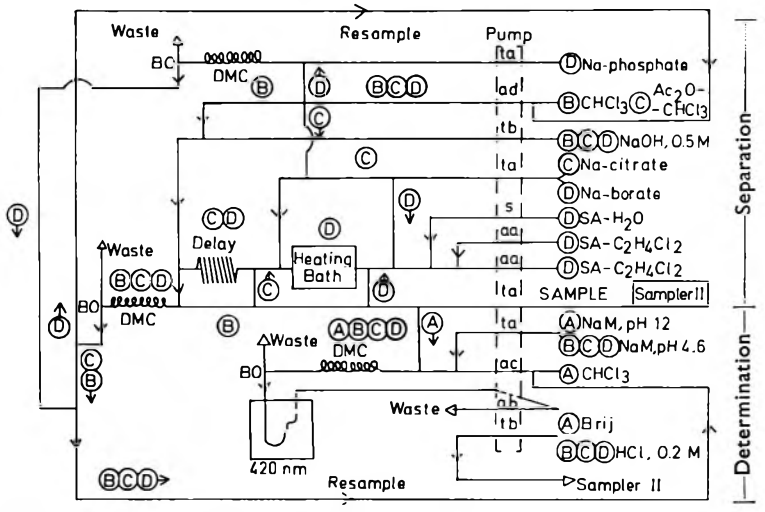

FIG. 2. Total manifold for selective determinations of ammonium ions by four separate runnings. Notes for reading the scheme: 1. Horizontally only readings from right to left should be made, except in the 2 cases of resampling. 2. Vertically the directions are shown by arrows. 3. The parts of the manifold used in each running are indicated by letters on or at the lines. Abbreviations: aa = Acidflex 0·056″; ab = 0·065; ac = 0·073; ad = 0·081; ta = Tygon 0·035; tb = 0·045; s = Solvaflex 0·056; DMC = Double Mixing Coil. Transport tubes and time delay coil are made of polyethene (bore 1·5 mm, wall 0·6 mm).

time delay coil and for the coupling between salicylic aldehyde and the primary amine both a heating bath and the time delay coil are used. In the present investigation no heating was necessary.

The rates of analysis are 40/h for runnings A and B, 30/h for running C and 20/h for running D. The Acidflex tubes are rinsed by pumping 0·1N HCl in ethanol followed by ethanol. Tygon and Solvaflex tubes are rinsed by water.

RESULTS

The determination step of the procedure (running A) was tested on the quaternary ammonium ions (pH 12 in the aqueous phase) and the amines (pH 4·6 in the aqueous phase) using samples containing only one substance. In all cases recoveries of 100·0% were obtained with a relative variation of about 1%. Results from tests of the masking procedures are given in Table 3. The acetylation procedure (running

Table 3. *Tests of the masking procedures in the automated method.* Standard: promethazine. The indications of the runnings refer to Table 2

Running	Sample	Concentrations × 10^4 added	found
C	Promethazine	1·60	1·60
	Dodecylamine	3·20	
D	Promethazine	0·310	0·317
	Dodecylamine	0·540	
D	Promethazine	0·350	0·707
	Protriptyline	0·350	
	Dodecylamine	0·350	

Table 4. *Complete analysis of ammonium compounds by the automated method*

Running	Determined components	Concentration × 10^5		Standard deviation	Number of determinations
		added	found		
A	*N*-hydroxyethyl-promethazine	6·21	6·34	0·10	30
C	Promethazine	7·00	7·14	0·05	30
B	Dodecylamine Protriptyline Promethazine	6·26	6·16	0·07	30
D	Protriptyline Promethazine	6·96	6·88	0·11	10

Sample: dodecylamine, 7·03 × 10^{-5}M; protriptyline, 6·91 × 10^{-5}M; promethazine, 7·00 × 10^{-5}M; *N*-Hydroxyethylpromethazine, 6·21 × 10^{-5}M. Standards: running A—*N*-hydroxyethyl-promethazine; running B and D—protriptyline; running C—promethazine. The sample is diluted in running B (3 + 7) and in running D (1+1).

Recoveries: dodecylamine 96%; protriptyline 96%; promethazine 102%; *N*-hydroxyethyl-promethazine 102%.

C) seems to give complete masking while the results obtained in running D which comprises the azomethine formation are too high. A complete analysis of a sample containing ammonium ions of four degrees of substitution is reported in Table 4.

The relative variation in each of the runnings is about 1% but the results of dodecylamine and protriptyline have a relative variation of about 2% since they are calculated as differences, dodecylamine from runnings B and D, protriptyline from runnings D and C. The deviations from 100% recovery are partly due to the slightly incomplete masking of the primary amine. They might, however, also be referred to the evaluation of several components by one single standard, since the compounds have been found to give slightly different peak forms.

Acknowledgements

The work has been supported by a grant from Apotekarsocieteten which is gratefully acknowledged. I also want to thank Drs Göran Schill and Olle Eriksson for interest and valuable discussions and AB KABI and Technicon, Sweden, for supplying me with laboratory equipment and materials.

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Determination of piribenzil in urine containing a metabolite and dextropropoxyphene by an ion pair extraction method

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Piribenzil, an antispasmodic quaternary ammonium compound, has been determined quantitatively in human urine containing a metabolite of piribenzil and dextropropoxyphene. Ion pair extraction with bromothymol blue as counter ion was used and the extraction conditions were calculated from extraction constants and partition coefficients determined using methylene chloride as organic solvent. The determination was made by photometry in the range 5–50 μg of piribenzil methylsulphate/ml of urine. The recovery was 97% $\pm$ 3%. The co-extraction of urine components such as nicotine, tyramine, tryptamine and choline was investigated by determination of constants for the partition of these compounds.

In this paper we have used the principles of Schill (1965) for quantitative determination in human urine samples of the antispasmodic quaternary ammonium compound piribenzil. The samples also contained dextropropoxyphene and a metabolite of piribenzil, 1,1-dimethyl-2-hydroxymethylpiperidinium. Piribenzil was isolated and determined photometrically in the concentration range 5–50 μg per ml urine by ion pair extraction with bromothymol blue.

The disturbance by other urine components like nicotine and the endogenous substances tyramine, tryptamine and choline has also been studied.

EXPERIMENTAL

Apparatus. The photometric determinations were made with a Zeiss Spektralphotometer PMQ II and the pH measurements with a Radiometer pH Meter 4.

Reagents and chemicals. All substances were of analytical grade. The methylene chloride was shaken with water before use. Bromothymol blue was purified according to Borg, Modin & Schill (1968). Sodium phosphate and sodium borate buffer solutions were used as aqueous phases.

Determination of the partition ratio. The partition studies were performed according to Modin & Schill (1967), using a shaking time of 20 min in centrifuge tubes at 20°. The phases were separated with a capillary siphon.

RESULTS AND DISCUSSION

Determination of extraction conditions

Piribenzil was determined in urine samples containing >5 μg/ml of the drug. The sample also contained an amine, dextropropoxyphene, at less than 10% of the piribenzil concentration. A metabolite of piribenzil, 1,1-di-methyl-2-hydroxymethylpiperidinium, was also assumed to be present to about the same concentration as piribenzil.

In the actual concentration range ($>5 \times 10^{-6}$M) extraction and photometric determination of piribenzil as ion pair with bromothymol blue could be assumed to give both good sensitivity and sufficient degree of extraction (Modin & Schill, 1967).

The method was based on constants for the extraction since these give the necessary information about selectivity which is of significance for a biological sample.

The extraction equilibrium can be defined by the extraction constant, E_{HAHY}

$$E_{HAHY} = \frac{[HAHY]_{org}}{[HA^+] \times [HY^-]} \quad .. \quad .. \quad .. \quad (1)$$

where A represents ammonium compounds and H_2Y is bromothymol blue. The extraction is influenced by side-reactions which are protolysis and partition of the protolytic ion pair components in uncharged form. Compensation for these side-reactions can be made if the partition coefficients ($k_{d(A)}$ and $k_{d(H_2Y)}$) and the acid dissociation constants (K'_{HA^+}, K'_{H_2Y} and K'_{HY^-}) or functions of these constants are known.

The calculation of this compensation has been demonstrated by Modin & Schill (1967). For the partition of the ammonium compound, A, as ion pair the following expression is valid

$$D_{HAHY} = \frac{[HAHY]_{org}}{C'_A} = E_{HAHY} \times C'_Y \times (\alpha_{HA} \times \alpha_{HY})^{-1} \quad .. \quad (2)$$

C'_A and C'_Y represent the concentrations of A and H_2Y not extracted as ion pairs.

$$\alpha_{HA} = 1 + K'_{HA^+} (1 + k_{d(A)}) \times (a_{H^+})^{-1} \text{ (monovalent amine)} \quad .. \quad (3)$$

$$\alpha_{HY} = 1 + a_{H^+} (1 + k_{d(H_2Y)}) \times (K'_{H_2Y})^{-1} + K'_{HY^-} \times (a_{H^+})^{-1} \quad .. \quad (4)$$

The constants necessary for the calculation of the α-coefficients and D_{HAHY} are given in Tables 1 and 2. The partition ratio will be directed by the concentration of

Table 1. *Partition coefficients for amines and bromothymol blue.* Organic phase: Methylene chloride. Aqueous phase: sodium phosphate buffer solutions with an ionic strength of 0·1. A represents amines and H_2Y is bromothymol blue

	$-\log k_{d(A)} \cdot K'_{HA^+}$	$\log k_{d(H_2Y)}/K'_{H_2Y}$	pK'_{HY^-}
Dextropropoxyphene ..	3·05	—	—
Nicotine	6·50	—	—
Tryptamine	9·19	—	—
Tyramine	10·90	—	—
Bromothymol blue ..	—	5·40*	7·12*

* Taken from Schill (1964) and Schill & Marsh (1963).

bromothymol blue (C'_Y) and pH of the aqueous phase. The relation between the partition ratios of the ammonium compounds studied and pH of the aqueous phase are given in Figs 1 and 2 ($C'_Y = 10^{-3}$). For the quaternary ammonium ions the variation in D_{HAHY} is entirely due to changes in α_{HY} since $\alpha_{HA} = 1$ for these aprotic compounds. When $D_{HAHY} > 100$ and equal phase volumes are used more than 99% of the ammonium compound is extracted as ion pair with bromothymol blue. From Fig. 1, it follows that piribenzil can be extracted quantitatively at pH <9.

Table 2. *Extraction constants for ion pairs with bromothymol blue.* Organic phase: methylene chloride. Aqueous phase: sodium phosphate buffer solution with an ionic strength of 0·1. HA^+ represents all kinds of ammonium ions

Cation	$[HAHY]_{org} \cdot 10^5$	log E_{HAHY}
Dextropropoxyphene	2·5 – 5·7	10·29
Piribenzil	1·3 –18	7·20
Dimethylhydroxymethylpiperidinium ..	0·39– 1·3	4·10
Choline		3·10*
Nicotine	1·3 – 2·3	5·82
Tryptamine	4·0 –10·1	4·39
Tyramine	0·70– 1·90	2·51

* Taken from Schill (1965).

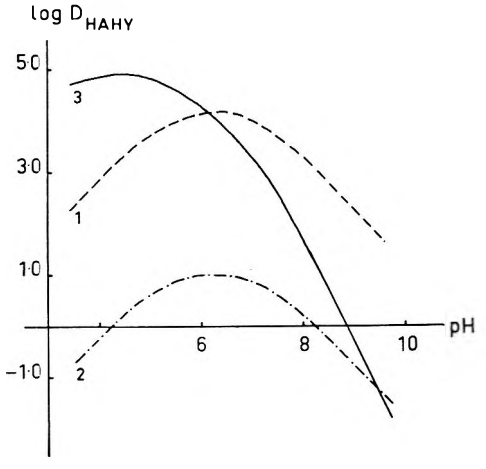

FIG. 1. Graphical illustration of the relation between the partition ratios of ion pairs with bromothymol blue and pH of the aqueous phase. 1. Piribenzil. 2. 1,1-Dimethyl-2-hydroxymethylpiperidinium. 3. Dextropropoxyphene.

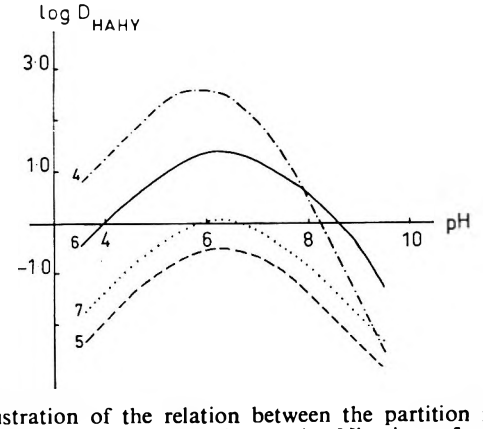

FIG. 2. Graphical illustration of the relation between the partition ratios of ion pairs with bromothymol blue and pH of the aqueous phase. 4. Nicotine. 5. Tyramine. 6. Tryptamine. 7. Choline.

The lower limit of the useful range is pH 7·5 since a lower pH will give a too high extraction of bromothymol blue in uncharged form.

The co-extraction of dextropropoxyphene and 1,1-dimethyl-2-hydroxymethyl-piperidinium have within the useful pH-range a minimum at pH 9 where both substances have $D_{HAHY} = 0{\cdot}1$ corresponding to a percentage extraction of 10%. A suitable way to reduce this co-extraction is to treat the organic phase once more with an aqueous phase of 10^{-3}M bromothymol blue at pH 9·0. The recovery of piribenzil will then be almost quantitative while the disturbance by the two other components in the piribenzil determination is $\leqslant 1\%$.

Quantitative determinations on urine samples

When urine samples were assayed under conditions giving quantitative extraction of piribenzil very high blanks were obtained. It was assumed that these blanks were due to the presence of other ammonium compounds with lower extraction constants than piribenzil, but present in the sample in much higher concentrations. In principle, compensation for these blanks was possible but they would decrease the precision of the quantitative determination of piribenzil considerably.

According to this assumption a re-extraction of the organic phase with an aqueous phase of 10^{-3}M bromothymol blue with pH 9·0 should decrease the blank value without significant losses of piribenzil (as discussed above). Blank values decreased in absorbance from 1·07 at the first extraction to 0·12 at the third extraction. The recovery of piribenzil after two repeated re-extractions was 97% while the blank value decreased about 10 times.

A blank value of a sample containing piribenzil can be obtained after hydrolysing piribenzil by alkali (Beckmann, 1966). The hydrolysing procedure is given in the *General procedure*. The hydrolysis gives 1,1-dimethyl-2-hydroxymethylpiperidinium which is extracted in a negligible amount as discussed above.

A method for analysis of piribenzil in urine samples based on the discussions above is given under *General procedure*. A test on samples containing 5–50 μg piribenzil-methylsulphate per ml gave a recovery of 97 $\pm$ 3% which is in good agreement with the theoretically calculated. Dextropropoxyphene and 1,1-dimethyl-2-hydroxy-methylpiperidinium were also present in the samples.

Co-extraction of other ammonium compounds

In the determination of blank values it was observed that smokers gave significantly higher values and also a larger daily variation. Since this effect was supposed to be due to co-extraction of nicotine, the extraction constant for the ion pair between bromothymol blue and nicotine was determined as well as the partition coefficient of nicotine. The constants are given in Table 1 and 2 and variation of the partition ratio with pH of the aqueous phase is demonstrated in Fig. 2. From these data it can be calculated that 2·5% of the total concentration of nicotine in urine will be extracted to the organic phase as ion pair with bromothymol blue.

Tables 1 and 2 and Fig. 2 also give data necessary for the calculation of disturbances due to co-extraction of tyramine, tryptamine and choline. The distribution ratios have sizes such that a disturbing co-extraction can be expected especially when these substances are close to the upper limit of the normal range of variation.

General procedure

Determination of piribenzil + blank. 5·00 ml of the urine sample is added to 5·00 ml of a sodium borate buffer solution ($C_{H_3BO_3}$ = 0·4M) with pH = 9·0 containing bromothymol blue in a concentration of $10^{-2.7}$. The extraction is with 10·00 ml methylene chloride in centrifuge tubes for 20 min. After centrifugation the organic phase is siphoned to another centrifuge tube containing 5·00 ml of the sodium borate buffer solution with bromothymol blue and 5·00 ml of water. Extraction is made as before and repeated once again. The organic phase from the third extraction is measured photometrically at 635 nm after the addition of a micro-drop of tetrabutylammonium hydroxide.

Determination of blank. To 5·00 ml of the urine sample 0·10 ml 10M NaOH is added. This solution is left for 20 min at room temperature. 0·10 ml 10M HCl is added and extraction and determination is performed according to the procedure above.

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Nuclear magnetic resonance spectroscopy of the trimethylsilyl ethers of some hydroxyphenylalkylamines*

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A number of hydroxyphenylalkylamines have been converted into their trimethylsilyl ethers by the action of hexamethyldisilazane in pyridine. The derivatives are completely soluble in carbon tetrachloride and these solutions are suitable for nuclear magnetic resonance spectroscopy. The nmr spectra of 16 potentially biologically active phenylalkylamines have been obtained in this way. The spectral data are presented and the significance of the results discussed.

In recent years nuclear magnetic resonance (nmr) spectroscopy has been widely used in medicinal and pharmaceutical chemistry for the identification and stereochemical investigation of biologically important molecules (cf. Review by Casy, 1967). However relatively little work has been reported dealing with nmr studies on phenylalkylamines in general and the catecholamines in particular.

The nmr spectrum of adrenaline (I) in D_2O/DCl was first discussed by Weiner, Pappas & Jardetzky (1961) and Weiner & Jardetzky (1964) in the course of their studies on the interaction of adrenaline and ATP. More recently the nmr spectra of adrenaline (I) and a number of other phenylalkylamines have been reported (Clauder, Radics & others, 1968; Reisch, Alfes & Möllmann, 1968). In addition to D_2O, trifluoroacetic acid and hexadeuterodimethylsulphoxide (containing trifluoroacetic acid) have been used as solvents in these studies. However all these solvents have certain disadvantages for use in nmr spectroscopy. Solutions of catecholamines in strong acids, such as trifluoroacetic acid, often undergo slow decomposition, whilst the inevitable proton resonance peaks due to HOD or incompletely deuterated dimethyl sulphoxide often tend to mask important spectral regions.

The twin problems of involatility and low non-polar solvent solubility often encountered when investigating both the chromatographic properties of hydroxylated phenylalkylamines and their behaviour in the mass spectrometer have often been overcome in the past by the formation of suitable derivatives. Several have been prepared for this purpose including acetyl derivatives (cf. Waldi, 1962; Brooks & Horning, 1964; Stern, Franklin & Meyer, 1967); trifluoroacetates (cf. Greer, Sprinkle & Williams, 1968; Kawai & Tamura, 1968) and trimethylsilyl derivatives (Sen & McGeer, 1963; cf. Review by Pierce, 1968). The possible use of the trifluoroacetates was not considered in the course of the present investigation since they are difficult to prepare in the pure state and tend to be unstable. Preliminary experiments made by the authors with tri- and tetra-acetyladrenaline (Prepared by the methods of Welsh, 1952, and Bretschneider, 1947, respectively) suggested that these acetyl derivatives would be unsuitable for nmr studies, since, despite their high solubility

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in non-polar solvents, their nmr spectra were unexpectedly complex, probably due to the existence of more than one conformation of the molecule in both cases.

The trimethylsilyl ethers of the catecholamines and related compounds are relatively easy to prepare and since this type of derivative has been successfully used in the past for nmr studies on other phenolic compounds, e.g. flavonoids (Waiss, Lundin & Stern, 1964), it was decided to investigate the usefulness of this type of derivative for nmr studies on the catecholamines and some related phenylalkylamines. The trimethylsilyl derivatives of hydroxyphenylalkylamines in which all the hydroxy groups are silylated are readly prepared by the action of hexamethyldisilazane on the amine in pyridine at 95° (cf. Review by Pierce, 1968).

EXPERIMENTAL

Phenylalkylamines

Commercial samples of the phenylalkylamine derivatives were used whenever possible. In some cases it was necessary to recrystallize the samples before use. If the sample was not available commercially it was prepared by one of the methods described in the literature. The following phenylalkylamines were used (the source or literature reference is indicated in parenthesis): adrenaline (Th. Schuchardt); noradrenaline hydrochloride, *N*-ethylnoradrenaline hydrochloride, *N*-isopropylnoradrenaline hydrochloride; metanephrine hydrochloride, phenylephrine hydrochloride (Winthrop Laboratories); *N*-n-butylnoradrenaline hydrochloride, synephrine (Regis Chemical Co.); epinine hydrochloride (Wellcome Research Laboratories); adrenaline methyl ether hydrochloride, adrenaline ethyl ether hydrochloride (Hukki & Seppäläinen, 1958); adrenaline n-butyl ether hydrochloride, β-*p*-hydroxyphenylepinine, β-3,4-dihydroxy-2-methylphenylepinine, adrepine, adnamine (Forrest, Kăspárek & others, 1969); O^3,O^4-dimethyladrenaline (a gift from Dr. B. Jaques).

Preparation of trimethylsilyl derivatives

Hexamethyldisilazane* (2·4 ml) was added to a suspension of the phenylalkylamine (free base or hydrochloride: 100 mg) in anhydrous pyridine (2 ml) in an atmosphere of nitrogen. The reaction flask was loosely stoppered and heated, with stirring, on an oil-bath at 80–100° for 30–60 min. In cases where the free base was used, the reaction mixture was heated for 5 min after a clear solution was obtained. The reaction mixture was cooled to room temperature, and if solid was present, filtered rapidly through a fine sintered glass funnel. The flask and residue were washed with dry carbon tetrachloride (B.D.H. "Analar" grade). The combined filtrate and washings were concentrated to dryness *in vacuo* below 30°. Residual pyridine was removed from the product, usually a pale yellow oil, by repeated evaporation with carbon tetrachloride (2 ml portions). A solution of the product, so obtained, in carbon tetrachloride (1 ml) was used directly for nmr analysis.†

Nuclear magnetic resonance spectroscopy

The nmr spectra were obtained on a Varian A-60-A instrument. Chemical shifts are reported as τ values. Tetramethylsilane was used as an internal reference. The spectra were recorded both in the presence and absence of the reference compound.

* In practice it was observed that the best results were obtained when fresh samples of this reagent were used.

† These solutions tend to decompose on storage.

This was necessary in view of the proximity of some of the peaks in the spectrum to the reference peak.

RESULTS AND DISCUSSION

The nmr spectra of the trimethylsilyl derivatives of 16 hydroxyphenylalkylamine derivatives (freshly prepared in carbon tetrachloride solution) were recorded and the spectral data are given in Table 1.

This method has proved to be very useful for obtaining the nmr spectra of a number of hydroxyphenylalkylamines, containing a secondary amino-group. However difficulties were encountered when primary amines were used. The fact that complex and variable spectra were obtained with the trimethylsilylated primary amines was probably due to partial or inconsistent silylation of the primary amino-group. This was not altogether surprising since the silylation reaction was carried out with hexamethyldisilazane in pyridine and incomplete silylation of primary amino-groups has previously been reported with this reagent mixture. A detailed consideration of the procedures available for the silylation of phenylalkylamines in general and catecholamines in particular is outside the scope of this paper. The subject has however been reviewed in a recent book by Pierce (1968).

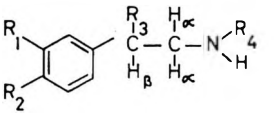
The following general conclusions can be drawn from consideration of the spectra of the trimethylsilyl derivatives of the 16 secondary hydroxyphenylalkylamines investigated. It was found that in all cases the hydroxyl groups were fully silylated but the secondary amino-groups were unaffected.

The aromatic ether trimethylsilyl protons were observed as sharp singlets in the region 9·75–9·83 τ, whilst the benzylic ether trimethylsilyl protons were seen at 9·94–9·99 τ, also as sharp singlets. The chemical shift of the trimethylsilyl protons appears to be a reliable indication of the type of hydroxyl group which has been silylated. The N-CH_3 group, when present, was observed in the range 7·59–7·62 τ, except for one of the N-CH_3 groups in adrepine (XV) and that in adnamine (XVI) which were found at 7·80 τ and 7·83 τ respectively. The N-CH_3 signal usually appeared as a sharp singlet, but extensive broadening of the N-CH_3 peaks was observed in two cases where the molecule contained a methoxyl group at the 3-position of the aromatic ring, i.e. metanephrine (VIII) and O^3,O^4-dimethyladrenaline (IX).

The N-H proton was observed as a broad signal in the 5·5–9·0 τ range; the position of the signal appeared to be markedly affected by concentration and temperature.

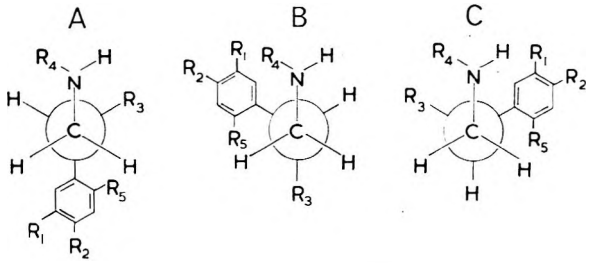
The positions and multiplicity of the signals due to the protons on the carbon atoms of the side-chain varied with the substitution pattern. In the compounds with a trimethylsilyl ether group on the carbon atom β to the nitrogen (i.e. I–IV; VIII–XI, XV) the signal due to the methine hydrogen [Hβ] on this carbon was observed between 5·23 and 5·42 τ, with a variety of different ring and N-alkyl substituents. In compounds with an O-alkyl group in this position (i.e. V, VI and VII), the corresponding methine proton signal was observed at a position about 0·5 ppm upfield in the range 5·75 to 5·85 τ. In compounds with either an alkyl or trimethylsilyl ether group in the β-position, the protons [Hα] on the carbon α to the nitrogen were seen between 7·30 and 7·43 τ, whereas in the β,β-diarylethylamine derivatives (i.e., XIII–XV), in which a second aryl group replaces the β-ether, these protons were seen in the 6·95–6·99 τ range. In the tricyclic compound adnamine the α proton is seen at 7·35 τ and the β proton at 6·22 τ.

Table 1. *Hydroxyphenylalkylamine trimethylsilyl ethers. Nuclear magnetic resonance spectra data*



Phenylalkylamine derivative	Compound No.	R_1	R_2	R_3	R_4	H_α	H_β	Notes
Adrenaline	I	9·77 (s)	9·77 (s)	9·99 (s)	7·61 (s)	7·43 (d), 7·39 (d) J = 5·0, J = 7·5	5·40 (dd) J = 5·0 & 7·5	R_1,R_2,R_3 = OTMS; R_4 = CH_3
N-Ethylnoradrenaline ..	II	9·78 (s)	9·78 (s)	9·99 (s)	CH_3CH_2 (see notes)	7·33 (d), 7·31 (d) J = 5·0, J = 7·0	5·38 (dd) J = 5·0 & 7·0	R_1,R_2,R_3 = OTMS; R_4 = $-CH_2$ $-CH_3$ 7·35 (q), 8·94 (t)
N-Isopropylnoradrenaline ..	III	9·79 (s)	9·79 (s)	9·99 (s)	$(CH_3)_2CH$ (see notes)	7·39 (d), J = 6·5,	5·42 (t) J = 6·5	R_1,R_2,R_3 = OTMS; R_4 = $(CH_3)_2$ $-CH$ 8·99 (d), 9·01 (d) 7·25 (sp)
N-n-Butylnoradrenaline ..	IV	9·78 (s)	9·78 (s)	9·99 (s)	$CH_2CH_2CH_2CH_3$ (see notes)	7·32 (m)	5·42 (dd) J = 5·0 & 7·0	R_1,R_2,R_3 = OTMS; R_4 = CH_2 - CH_2CH_2 - CH_3 9·17 (t), 8·70 (m), 7·47 (t)
Adrenaline methyl ether ..	V	9·77 (s)	9·77 (s)	6·71 (s)	7·60 (s)	7·39 (d), 7·33 (d) J = 5·0, J = 8·0	5·85 (dd) J = 5·0 & 8·0	R_1,R_2 = OTMS; R_3 = OCH_3; R_4 = CH_3
Adrenaline ethyl ether ..	VI	9·75 (s)	9·75 (s)	OCH_2CH_3 (see notes)	7·61 (s)	7·42 (d), 7·35 (d) J = 5·0, J = 8·0	5·75 (dd) J = 5·0 & 8·0	R_1,R_2 = OTMS; R_4 = CH_3; R_3 = $-O-CH_2$ $-CH_3$ 6·64 (q), 6·67 (q), 8·84 (t)
Adrenaline n-butyl ether ..	VII	9·75 (s)	9·75 (s)	O-*n*-butyl (see notes)	7·61 (s)	7·40 (d), 7·33 (d) J = 5·0, J = 8·0	5·77 (dd) J = 5·0 & 8·0	R_1,R_2 = OTMS; R_4 = CH_3; R_3 = CH_3-CH_2CH_2-CH_2O 9·12 (t), 8·55 (m), 6·68 (m)
Metanephrine	VIII	6·22 (s)	9·78 (s)	9·98 (s)	7·61 (bs)	7·42 (bm)	5·33 (t) J = 6·0	R_1 = OCH_3; R_2,R_3 = OTMS; R_4 = CH_3
O^3,O^4-Dimethyladrenaline ..	IX	6·23 (s)	6·20 (s)	9·98 (s)	7·61 (bs)	7·35 (bm)	5·29 (t) J = 6·5	R_1,R_2 = OCH_3; R_3 = OTMS; R_4 = CH_3
Synephrine	X	H (aromatic)	9·76 (s)	9·99 (s)	7·59 (s)	7·30 (m)	5·23 (t) J = 6·5	R_1 = H; R_2,R_3 = OTMS, R_4 = CH_3 Aromatic AA'BB' at 3·09, J = 9·0
Phenylephrine	XI	9·78 (s)	H aromatic	9·97 (s)	7·62 (s)	7·39 (d), 7·41 (d) J = 7·0, J = 5·0	5·31 (dd) J = 5·0 & 7·0	R_1 = OTMS; R_2 = H; R_3 = OTMS; R_4 = CH_3
Epinine	XII	9·79 (s)	9·79 (s)	7·33 (bt)	7·62 (s)	7·33 (bt)	7·33 (bt)	R_1,R_2 = OTMS; R_3 = H; R_4 = CH_3

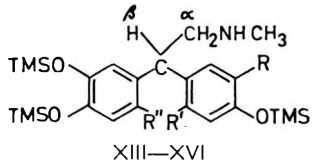
Notes: The aromatic proton signals occurred as multiplets in the region of 3·09 to 3·34.
The position of the N–H signal was concentration dependent and was found within the range of 5·57 to 8·95.
(s) = singlet; (d) = doublet; (dd) = doublet of doublets;
(t) = triplet; (q) = quartet; (b) = broad; (sp) = septet; (bs) = broad singlet.



The conformational populations of the various compounds could be estimated from the coupling constants of the side-chain protons which depend upon the percentage distribution of the individual conformers (cf. Emsley, Feeney & Sutcliffe, 1965). Values for the *trans* and *gauche* coupling constants, required for these calculations were obtained from consideration of the spectrum of adrepine [XV] (Forrest, Kăspárek & others, 1969). This amine which has an extra bulky substituent [R_5] in the *ortho*-position, causing it to exist mainly as one of conformer A or B, shows a *trans* coupling of 9·0 Hz (dihedral angle of 180°) and a *gauche* coupling of 2·5 Hz (dihedral angle of 60°). Using these values for the *gauche* and *trans* coupling constants of all conformers, estimates of the conformer populations could be made. The approximate distribution was found to vary from a 50 : 50 mixture of A and B, as in the case of *N*-isopropylnoradrenaline [III], to a 70 : 30 mixture as in the case of adrenaline [I]. It must be emphasized that in view of the first order analysis of the spectra and the use of *trans* and *gauche* coupling constants from a model compound, these values must be considered as approximations only.

This procedure for determining the nmr spectra of catecholamines should prove a useful adjunct to mass spectrometry and gas liquid chromatography for the identification of this type of compound.

Table 2. *Diphenylalkylamine trimethylsilyl ethers. Nuclear magnetic resonance spectral data*



XIII—XVI

Diphenylalkylamine derivative	Compound number	Aromatic OTMS	N–CH_3	Hα	Hβ	Aromatic H	Others
β-*p*-Hydroxyphenylepinine R = R′ = R″ = H	XIII	9·77(s) 9·79(s) 9·83(s)	7·59(s)	6·95(d) *J* = 7·5	6·00(t) *J* = 7·5	3·29(m) 3·08 AA′BB′ *J* = 9·0	
β-3,4-Dihydroxy-2-methylphenylepinine R = OTMS; R′ = CH_3, R″ = H	XIV	9·78(s) 9·87(s)	7·60(s)	6·96(d) *J* = 7·0	5·83(t) *J* = 7·0	3·30(m)	C–CH_3 7·87(s)
Adrepine R = OTMS; R′ = C(OTMS)(α′)–CH_2–NHCH_3(β′); R″ = H	XV	9·82(s) 9·79(s) 9·77(s) 9·74(s)	7·59(s) 7·80(s)	6·99(d) *J* = 7·0	5·74(t) *J* = 7·0	3·29(m)	Aliphatic OTMS 9·94(s) Hα′ 8·12(dd), *J* = 12·0 and 2·5 Hα′ 7·47(dd), *J* = 12·0 and 9·0 Hβ′ 4·99(dd), *J* = 2·5 and 9·0
Adnamine R = OTMS; R′–R″ = –CH=CH–	XVI	9·83(s) 9·81(s)	7·83(s)	7·35(d) *J* = 7·5	6·22(t) *J* = 7·5	3·44(bs)	H olefinic 3·44(bs)

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An investigation of the distribution coefficients of some androgen esters using paper chromatography

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R_M values have been determined for four homologous series of androgen esters, using a Bush paper chromatography system, to assess the value of the ratio of the solubilities in the individual solvents (solubility ratio), as an estimate of distribution coefficient. The distribution coefficients were proportional to R_M values in all four series, and for three of these the proportionality constant was the same. Limitations in the use of R_M values to predict biological effects have been pointed out.

James, Nicholls & Roberts (1969) have observed a relation between ethyl oleate-water distribution coefficients and biological half life for testosterone esters. Distribution coefficients were quoted as the ratio of the solubilities in the individual solvents (solubility ratio). This is valid provided both saturated solutions are dilute and the mutual solubilities of the solvents are negligible, but it is questionable whether the distribution coefficients of steroid esters, between organic solvents and water would satisfy these requirements. It was therefore considered necessary to confirm the distribution coefficients by another method.

Paper chromatography is essentially a liquid–liquid distribution process. Bate-Smith & Westall (1950) have shown that

$$\log \alpha = R_M + k \qquad (1)$$

where

$$R_M = \log\left(\frac{1}{R_f} - 1\right) \qquad (2)$$

α is the distribution coefficient between moving and stationary phases and k is a constant, dependent on the ratio of the volumes of stationary and moving phases. R_M values have been used to estimate distribution coefficients by Iwasa, Fujita & Hansch (1965), and Boyce & Milborrow (1965) correlated R_M values of *N*-alkyltritylamines with moluscicidal activity, which is dependent on distribution coefficients.

A further objection to the use of solubility ratios to estimate distribution coefficients is the difficulty of determining aqueous solubilities of steroids, which are frequently too low to give reliable spectrophotometer readings. We have measured the solubilities and R_M values of a series of steroid esters on paper to assess the value of the ratio of the solubilities as an estimate of their distribution coefficients, and to develop a simpler method of determining the distribution coefficients of steroids.

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EXPERIMENTAL

Preparation of esters

Formate esters were prepared according to Ringold, Loken & others (1957) (preparation of Δ^5-androsten-3β-ol-17-one formate) and recrystallized from n-hexane. The remainder were obtained by refluxing with the anhydride in the presence of pyridine, and recrystallized from aqueous ethanol. Melting points, where published, agreed with the literature.

5,6-*Dehydroisoandrosterone* (3β-hydroxyandrost-5-ene-17-one) *formate*. $[\alpha]_D^{20}$ + 10·5 (*c* 3·6 in EtOH). M.p. 140°; Fajkos (1959) gave 140–141°.

5,6-*Dehydroisoandrosterone acetate*. $[\alpha_D^{20}]$ + 14·9 (*c* 1·9 in EtOH). M.p. 167°; De Ruggieri & Ferrari (1959) gave 167–170°.

5,6-*Dehydroisoandrosterone propionate*. White crystals, $[\alpha]_D^{20}$ + 14·7 (*c* 1·6 in EtOH). M.p. 197°; Dr J. L. Marsh, in a personal communication, gave near 198°. Found: C, 76·4; H, 9·8. $C_{22}H_{32}O_3$ requires: C, 76·7; H, 9·3.

5,6-*Dehydroisoandrosterone butyrate*. White crystals, $[\alpha_D^{20}]$ + 13·1 (*c* 1·8 in EtOH). M.p. 163°. Found: C, 76·6; H, 9·9. $C_{23}H_{34}O_3$ requires: C, 77·05; H, 9·6.

Oxime. White crystals, m.p.150°. Found: C, 73·3; H, 9·25; N, 3·8. $C_{23}H_{35}NO_3$ requires: C, 74·0; H, 9·4; N, 3·75.

5,6-*Dehydroisoandrosterone valerate*. White crystals, $[\alpha]_D^{20}$ + 13·1 (*c* 1·8 in EtOH). M.p. 120°. Found: C, 78·3; H, 9·0. $C_{24}H_{36}O_3$ requires: C, 77·4; H, 9·7.

Oxime. White crystals, m.p. 160°. Found: C, 75·0; H, 9·55; N, 3·35. $C_{24}H_{37}NO_3$ requires: C, 74·4; H, 9·6; N, 3·6.

Nuclear magnetic resonance spectra for propionate, butyrate and valerate gave peaks at τ 4·55, equivalent to one olefinic proton which would therefore be in position 4, 6, 7 or 11. Klyne (1957) has shown that the optical rotations of steroids are affected by the presence and position of olefinic bonds, and quoted the following increments for substitution of a double bond for a single bond: Δ^4 + 194°; Δ^5 − 298°; 5α,Δ^7 − 68°; 5β,Δ^7 + 119°; 5α,$\Delta^{9(11)}$ + 109°; 5β,$\Delta^{9(11)}$ + 49°. The specific rotations found for propionate, butyrate and valerate agree with those for formate and acetate, which are known compounds, and with $[\alpha]_D^{20}$ + 10·0° (in EtOH) quoted for the parent alcohol (Lang, 1961). It is therefore concluded that the double bond is in the 5,6 position in all the esters, since a movement of the unsaturation to another position would bring about a large change in specific rotation.

Testosterone decanoate was a gift from Organon Laboratories Ltd.

Chromatography

Preliminary experiments established that the Bush (1961) system, formic acid–methanol–light petroleum (85–90°) (100:90:10) was suitable, yielding Rf values between 0·15 and 0·75 with most of the compounds examined, and gave measurable differences between esters without streaking. Whatman No. 1 paper was spotted with 1·0 μl of a 5% solution of the pure steroid, using a microlitre syringe, and equilibrated with the stationary phase and moving phase in a tank placed in a constant temperature cupboard at 25° for at least 3 h, usually overnight. The length of run, the position of the starting line and volume of mobile phase added were kept constant. Development was carried out using the descending technique and the spots detected by spraying with 2,4-dinitrophenylhydrazine, except those of

the 5,6-dehydroisoandrosterone esters which were detected with alkaline *m*-dinitrobenzene.

Solubility determinations

The solubilities of the testosterone esters in water and organic solvents have been reported previously (James & Roberts, 1968). The same techniques were used to prepare saturated solutions of the remaining compounds and to determine their solubilities in cyclohexane. Saturated solutions in water, of androstanolone and its esters were extracted with n-hexane and assayed colorimetrically using 2,4-dinitrophenylhydrazine (Jordan & Veatch, 1964).

RESULTS AND DISCUSSION

The solubilities of the formate to valerate esters of testosterone in organic solvents change irregularly as the series is ascended, the acetate appearing particularly anomalous. This behaviour has been linked to the melting points by James & Roberts (1968) and reasons suggested for the changes in solubility. The analogous androstanolone esters behave differently. Both profiles are shown in Fig. 1A. Aqueous solubilities increased logarithmically with addition of each CH_2 in both

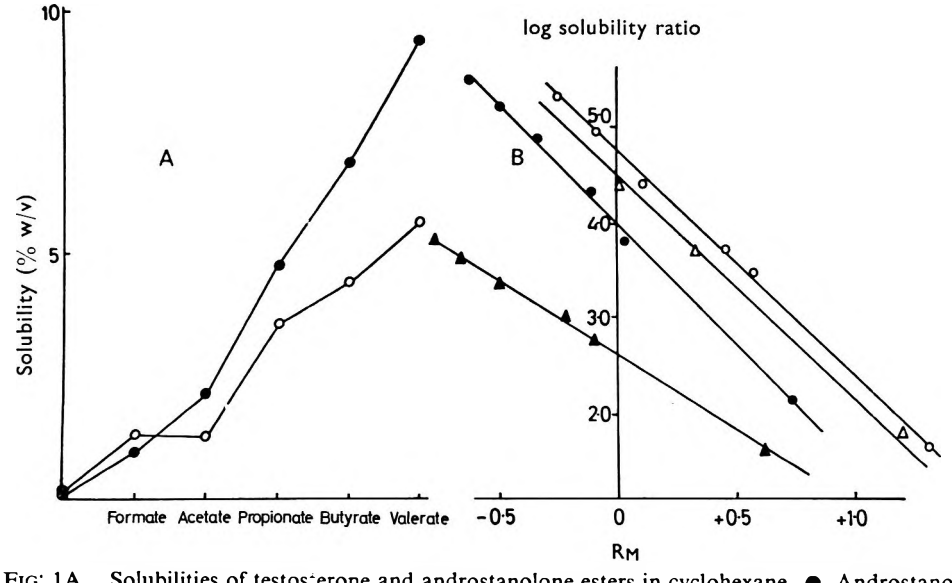

FIG. 1A. Solubilities of testosterone and androstanolone esters in cyclohexane. ●, Androstanolone series; ○, testosterone series. B. Relations between R_M value and log solubility ratio (ordinate). ○, Testosterone series; △, methyltestosterone series; ●, androstanolone series; ▲, 5,6-dehydroandrostanolone series.

series. Solubilities in cyclohexane and water are given in Table 1, together with R_M values for the formic acid–methanol–light petroleum system. Plots of solubility ratio cyclohexane-water against R_M were linear for both series. These are shown in Fig. 1B, and are typical of all solvents examined. The slopes of the two lines are not significantly different ($P = 0{\cdot}99$), having a mean slope of $-2{\cdot}40$, despite the fact that the testosterone and androstanolone series give different solubility profiles in

Table 1. *Solubilities and R_M values*

Compound	Solubility, % w/v Cyclohexane	Water × 10^5	R_M value
Testosterone	0·088	196	1·31*
Formate	1·24	44·0	0·58 ± 0·06
Acetate	1·18	23·5	0·46 ± 0·04
Propionate	3·55	14·8	0·11 ± 0·03
Butyrate	4·41	5·03	—0·09 ± 0·03
Valerate	5·71	2·91	—0·26 ± 0·02
Androstanolone	0·069	52·5	0·74*
Formate	0·891	14·9	0·03 ± 0·04
Acetate	0·210	9·75	—0·11 ± 0·04
Propionate	0·479	6·20	—0·34 ± 0·03
Butyrate	0·694	4·40	—0·50 ± 0·03
Valerate	0·949	3·05	—0·63 ± 0·03
17α-Methyltestosterone	0·145	226	1·20 ± 0·03
Acetate	0·881	17·9	0·33 ± 0·02
Propionate	2·448	10·2	0·01 ± 0·03
5,6-Dehydroisoandrostorone	0·106	249	—
Formate	0·758	140	—0·10 ± 0·03
Acetate	1·10	115	—0·22 ± 0·03
Propionate	1·83	83·2	—0·50 ± 0·04
Butyrate	3·17	79·0	—0·67 ± 0·03
Valerate	4·79	76·8	—0·78 ± 0·04

* Calculated by Socziwinski's method.

organic solvents. This does not prove that the solubility ratio gives the true distribution coefficient, but it can be inferred that it varies from ester to ester in the same way as the distribution coefficients. A third plot, for 17α-methyltestosterone and its acetate and propionate is also shown and suggests that this series parallels the other two. The use of solubility ratios in comparing distribution coefficients with biological activity therefore appears to be justified.

Collander (1951) has shown that, for two solvent systems A and B,

$$\log \alpha_A = a \log \alpha_B + b \qquad (3)$$

where a and b are constants characteristic of the solvent systems used. The constant slope for the androstanolone, testosterone and methyltestosterone series in Fig. 1B supports equation (3) and suggests that, given the distribution coefficient of one ester, those of its homologues can be estimated from R_M values using the equation

$$\log (\text{unknown } \alpha) = \log (\text{known } \alpha) - 2{\cdot}4 \times (R_{M\,(\text{known})} - R_{M\,(\text{unknown})}) \qquad (4)$$

The equation is limited in its scope, however, thus testosterone decanoate gave a point above the line in the plot of R_M against solubility ratio, indicating that equation (4) does not extend indefinitely up the homologous series. Deviations have been observed with the higher members of other homologous series, by Trzaska & Kowkabany (1967), and attributed to the increasing influence of adsorption as the size of the alkyl chain increased.

The R_M values for androstanolone and testosterone did not agree with those predicted from Fig. 1B, but were identical with those for the corresponding formate esters. The spots were eluted from the paper with ethanol, and the residues, obtained after evaporating off the ethanol, taken up in carbon tetrachloride. The infrared spectra of these solutions were characteristic of esters, neither absorbed in the -OH stretching region, but both gave an ester C=O peak at 1740 cm^{-1}, suggesting

that formylation had occurred on the paper. Methyltestosterone was not formylated, probably because of the hindrance of the 17α-methyl group.

Theoretical R_M values for testosterone and androstanolone were estimated by Socziwinski's method (1965), using acetic acid–light petroleum. This did not acetylate the steroid alcohols, but was otherwise less satisfactory than the formic acid system, as marked streaking occurred. The calculated R_M values fitted the results in Fig. 1B.

A further limitation of equation (4) can be seen from the results for the esters of 5,6-dehydroisoandrosterone, given in Fig. 1B. R_M values are linearly related to solubility ratios, but the slope is significantly less than that for the other three series. Application of equation (4) would therefore lead to incorrect conclusions if applied to the 5,6-dehydroisoandrosterone series. If A represents the solvent system in a chromatographic process and B the solvent system with which it is compared, equations (1) and (3) can be combined to give

$$\log \alpha_B = a.R_{MA} + ak + b \quad .. \quad .. \quad .. \quad .. \quad (5)$$

a, b and k are responsible for slopes and intercepts in Fig. 1B. If these were constant, as required by equations (1) and (3), there would be no variation between homologous series and the plots in Fig. 1B would be superimposable. A dependence of a and b on the nature of the solute was noted by Collander (1951) and attributed to the number of hydrophilic groups in the solute molecule and their effect on hydrogen bonding. The deviations noted here must be due to structural differences, since testosterone and 5,6-dehydroisoandrosterone have the same functional groups in the steroid nucleus but while 17α-methyltestostosterone and androstanolone are 17β-hydroxy-3-one steroids, 5,6-dehydroandrosterone is a 3β-hydroxy-17-one steroid. Several workers, notably Hansch, Muir & others (1963) have based their conclusions on equation (3), assuming that changes in the distribution coefficients in the solvent system used *in vitro* would be the same as those *in vivo*. It appears that this assumption must be treated with caution, particularly when applied to steroid molecules.

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The effects of temperature on the critical micelle concentrations of alkyl α-picolinium bromides

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The temperature dependence of the critical micelle concentrations of decyl, dodecyl and tetradecyl α-picolinium bromides has been determined by measurement of conductance. Constants for the equation relating log. CMC and alkyl chain length have been calculated for the temperature range 5° to 70°, in increments of 5°. An estimate of the effect of temperature upon the degree of counterion binding is recorded. Thermodynamic parameters have been calculated using the uncharged phase-separation model and a theoretical interpretation of the process of micellization given.

The enthalpy change, ΔHm, accompanying the aggregation of surfactant monomers into micelles may be determined by calorimetry (Goddard, Hoeve & Benson, 1957; White & Benson, 1959; Benjamin, 1964, 1966; Corkill, Goodman & Tate, 1964; Corkill, Goodman & others, 1966) or from the temperature dependence of the critical micelle concentration (CMC) (Flockhart & Ubbelohde, 1953; White & Benson, 1959; Hermann, 1962; Ingram & Jones, 1969).

A limitation to the calorimetric method is that with surfactants having dodecyl or longer alkyl chains the CMC has a low value. Consequently the total heat evolved upon the destruction of micelles in such dilute solutions is small, thereby putting a strain on the sensitivity of measurement. As a result many workers have been obliged to use short-chain compounds, which have higher CMCs and give more readily detectable heat changes on dilution of their solutions from above to below the CMC. Such measurements often do not agree with the enthalpies of micellization derived from the temperature dependence of CMC. This is to be expected as the CMCs are of such magnitude that it is probably unjustified to consider that the activities of such solutions can be replaced by concentrations. Recent measurements of ΔHm for sodium dodecyl sulphate by Pilcher, Jones & others (1969), using a twin differential calorimeter, would appear to have an accuracy greater than that of previous reports.

Because of the difficulties of attaining a high degree of accuracy using the calorimetric method, many workers have preferred to use the indirect method of obtaining ΔHm from CMC values. Results obtained in this laboratory for certain cationic surfactants have been previously published (Adderson & Taylor, 1964, 1967).

The present results extend these data to a homologous series.

EXPERIMENTAL

Materials

Decanol (purissima grade, Fluka A. G.) of greater than 99·5% purity as determined by gas-liquid chromatography, dodecanol and higher homologues absent.

Dodecanol (purissima grade Fluka A. G.) of greater than 99% purity, higher homologues absent, up to 1% of decanol present.

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Tetradecanol (purissima grade, Fluka, A. G.) of greater than 99·5% purity. Up to 0·25% of an unidentified contaminant having a longer retention time, when examined by gas-liquid chromatography, than tetradecanol but shorter than hexadecanol.

α-Picoline, Hopkins and Williams laboratory reagent, was fractionally distilled twice and the fraction distilling at 128° collected. A gas-liquid chromatogram showed the absence of the β- or γ-isomers.

Other reagents of Analar or laboratory reagent quality.

The alcohols were converted to the corresponding alkyl bromides (Organic Syntheses, 1935). Equimolar quantities of alkyl halide and α-picoline were refluxed on a boiling water bath for 12 h. The reaction products were agitated with light petroleum (40–60°) to remove unchanged alcohol or halide. The solid was separated and dissolved in a mixture of 80% methyl isobutyl ketone and 20% acetone and allowed to crystallize at 0°. This was repeated twice. The surfactants were continuously extracted with light petroleum (40–60°) for 12 h and then twice re-crystallized from a mixture of 10% methanol and 90% sodium-dried ether.

An elementary analysis was performed for the decyl homologue for which no melting point was available in the literature. Equivalent weights were determined by a non-aqueous titration of the bromide with perchloric acid in glacial acetic acid in the presence of mercuric acetate.

Bromine content was determined by coulometric analysis.

Decyl α-picolinium bromide: m.p. 117°C, equiv. wt found 314·5, calculated 314·22. Found: C, 61·2; H, 9·0; Br, 25·5; N, 4·5; calculated: C, 61·1; H, 9·0; Br, 25·4; N, 4·5%. No optical effects characteristic of liquid crystals were observed on heating to 140° or on subsequent cooling.

Dodecyl α-picolinium bromide: m.p. 124°C (124°, Kolloff, Wyss & others, 1942), equiv wt, found 346·4, calculated 342·37. Found: Br, 23·3; calculated, 23·6%. No optical effects as of liquid crystals observed up to 160°.

Tetradecyl α-picolinium bromide: m.p. 125 and 192°C (125, 192°C, Kolloff, Wyss & others, 1942), equiv wt, found 370·9, calculated 370·43. Found: Br, 21·6; calculated 21·4%. The lower melting point, corresponding to the transition to a non-mobile translucent gel, was extremely sensitive to moisture and required storage of the compound for several days above phosphorus pentoxide to give a consistent value. In plane-polarized light the gel exhibited characteristic effects as of liquid crystals some 30° above the temperature of transition from solid to a gel. On complete melting at 192° and subsequent cooling Grandjean terraces were observed.

The surface tension–concentration curves did not exhibit minimum values with subsequent increase to constant values.

CMC determinations

CMC values were determined according to Adderson & Taylor (1964) using ion-exchange water equilibrated with laboratory air ($K = 1{\cdot}91 \times 10^{-6}$ ohm^{-1} cm^{-1}).

RESULTS AND DISCUSSION

CMC values were calculated in terms both of mol fraction (Table 2) and as molalities. The latter system was used to test the application of the equation

$$\text{Log CMC} = A + BM \text{ (Table 2)}$$

where A and B are constants and M the number of carbon atoms in the principal alkyl chain. The constants A and B were obtained by a least squares treatment of the CMC data (Table 1).

Table 1. *Constants for the equation Log* CMC (molal) $= A + BM$

Temperature °C	A	B
10	1·842	−0·3175
15	1·809	−0·3151
20	1·788	−0·3134
25	1·772	−0·3118
30	1·756	−0·3098
35	1·737	−0·3073
40	1·715	−0·3040
45	1·689	−0·3003
50	1·661	−0·2961
55	1·634	−0·2918
60	1·608	−0·2875
65	1·587	−0·2835
70	1·572	−0·2801

The values of the constant B are similar to those found for alkyl trimethylammonium bromides and alkyl benzyl dimethyl ammonium bromides (this laboratory) suggesting that at any one temperature the addition of a further methylene group would have a similar effect upon the CMC values, and upon ΔG_m°, the free energy change upon micellization, for any of these homologous series.

The predicted constants gave CMC values that differed by up to 8·5% from the experimental values obtained. This is in contrast to the results of a similar treatment of the CMC values of alkyl trimethyl ammonium bromides (this laboratory, to be published). This lack of agreement for alkyl α-picolinium bromides may be a reflection of the total hydrocarbon content of the surfactants. A similar result has been noted for compounds having long straight hydrocarbon chains whereas shorter homologous compounds obeyed the above equation (Evans, 1956).

Table 2. *Critical micelle concentrations, mol fractions*

Temperature °C	Decyl	Dodecyl	Tetradecyl salt
5	$8{\cdot}74 \times 10^{-4}$	—	—
10	8·59	$1{\cdot}87 \times 10^{-4}$	$4{\cdot}60 \times 10^{-5}$
15	8·43	1·84	4·61
20	8·30	1·83	4·66
25	8·32	1·84	4·70
30	8·36	1·86	4·88
35	8·56	1·19	5·05
40	8·81	2·01	5·31
45	9·00	2·08	5·71
50	9·33	2·15	6·13
55	9·71	2·28	6·57
60	10·1	2·39	7·19
65	10·6	2·55	7·73
70	11·0	2·71	8·48

The CMCs have minimum values at specific temperatures; these temperatures are chain length dependent. Similar effects have been observed in alkyl trimethylammonium, alkyl dimethylbenzylammonium and alkyl pyridinium bromides (Adderson & Taylor, 1964 and unpublished data of this laboratory). Decyl cationic surfactants have a minimum CMC at approximately 23–25°, dodecyl at 18–20° and

tetradecyl at 10°. Emmerson & Holtzer (1967) have suggested that the minimum is the resultant of two opposing effects, namely the decrease in dielectric constant of water with increase in temperature, which increases the electrostatic repulsion of the heads, and a hydrocarbon effect. The latter is postulated as arising from the occurrence of "water-cages" stabilized by the hydrocarbon chain, the degree of structured water decreasing with temperature increase.

Butler, Stead & Taylor (1969) consider the observed minimum value of the CMC to be an artifact. In systems in which dimers have been detected in co-existence with monomers, the use of the monomer concentration only in the calculation of CMC values removes the conventional minimum value. It is, however, noteworthy that minimum values appear in the temperature dependence of the CMC of decyl compounds whereas specific investigations to detect dimers have not produced supporting evidence for their existence in such compounds (Mysels & Kapauan, 1961; Clunie, Goodman & Symons, 1967).

Evans (1956) has suggested that the charge on the micelle may be obtained from the specific conductance–concentration plots using

$$\frac{p^2}{N^{4/3}}(1000\ S_1 - \Lambda_R) + \frac{p\Lambda_R}{N} = 1000\ S_2$$

where p = the effective charge on the micelle, N = the aggregation number, Λ_R = the equivalent conductance of the counterion at infinite dilution, $S_1 = (dK_1/dC_1)$ = the slope of the specific conductance–concentration plot below the CMC, $S_2 = (dK_2/dC_2)$ = the similar slope above the CMC.

The concentrations in the present work have been expressed in terms of weight in weight, whereas Λ_R relates to a weight in volume relation. To correct the experimental measurements for this discrepancy the slopes dk/dc, with c (the concentration) in molal units, have been adjusted by a factor 1/D where D is the density of water at the requisite temperature. The difference between the density of water at 25°C and the most concentrated solution used is less than 0·1%.

The aggregation numbers N for alkyl α-picolinium bromides are not known. However, Evans has shown that p/N is insensitive to change in N. It is commonly observed that an increase in alkyl chain length is accompanied by an increase in N, for example for alkyl trimethylammonium bromides $N_{C10} = 36$, $N_{C12} = 50$, $N_{C14} = 75$ (Debye, 1949).

Limited values are available for alkyl pyridinium bromides; $N_{C11} = 42$, $N_{C14} = 79$ (Trap & Hermans, 1955). It seems reasonable therefore to assume that alkyl α-picolinium bromides would exhibit similar values and, for the purposes of subsequent calculations, values of N of 36 for the decyl, 50 for the dodecyl and 75 for the tetradecyl salts have been assumed.

The use of an invariant aggregation number has only a slight effect upon p/N. If N = 50 is assumed for all the homologues at 25°C, p/N becomes $C_{10} = 0{\cdot}29$, $C_{12} = 0{\cdot}22$ and $C_{14} = 0{\cdot}21$ so that the observed relations are scarcely altered. The use of a gradually decreasing value of N, such as would be expected with rise in temperature (Debye, 1949; Trap & Hermanns, 1955) enhances the increase in p/N with temperature. For the decyl compound at 55° if N = 32, p/N = 0·34. Thus the degree of dissociation increases, as expected with temperature. The extent of counterion binding increases in the order decyl<dodecyl<tetradecyl compound (Table 3).

A similar result has been observed in the alkyl trimethylammonium bromides (this laboratory), and has been reported for anionic surfactants (Evans, 1956).

Table 3. *The fraction of the theoretical charge, p/N, of alkyl α-picolinium bromides*

Temperature °C	Λ°Br (Robinson & Stokes, 1959)	Decyl, p/N	Dodecyl, p/N	Tetradecyl, p/N
15	63·1 ohm^{-1} cm^2	0·31	0·21	0·19
25	78·1	0·30	0·22	0·20
35	94·0	0·32	0·23	0·21
45	110·7	0·33	0·24	0·21
55	125·4	0·33	0·25	0·21

Thermodynamic parameters

If the micelle is treated as a separate phase the free energy change on micellization may be obtained by $\Delta G^\circ_m = (2 - \alpha)$ RT ln CMC. Variable values for ΔG° may be derived depending upon the definition of the composition of the phase.

If the phase is considered to consist of the micelles plus bound counterions, "the charged phase model" (Matijevic & Pethica, 1958; Shinoda & Hutchinson, 1962), somewhat lower values are obtained than if the phase is assumed to include all the counterions, whether bound or free "the uncharged phase model". In the latter case α, i.e. p/N as previously defined, is zero.

Values of p/N vary depending upon the method of determination. Estimates using light-scattering techniques (Mysels, 1955), or conductance slopes give values of 0·2 to 0·3, whereas estimates derived from the slopes of the graphs relating CMC values to electrolyte concentration are approximately 0·5 (Shinoda, 1963). With the present uncertainty of choice concerning this parameter, the uncharged model gives as valuable information as the charged model (Table 4).

Using a standard state of a mol fraction of unity for the monomeric species, and the micelle itself as the standard state for its species, and assuming that the heat of dilution from the standard state to the CMC is negligible compared to the heat of micellization, values may be calculated: $-\Delta G^\circ_m = -2$ RT ln CMC; $-\Delta H_m = 2\,RT^2\,(\partial \ln \text{CMC}/\partial T)_P$; $-\Delta S_m = -\Delta H_m/T$; $-\Delta S^\circ_m = (\Delta G^\circ_m - \Delta H^\circ_m)/T$.

Two entropy values may be calculated, ΔS°_m with respect to the change at the standard state, and ΔS_m with respect to the change at CMC. The difference between these values gives the almost constant entropy of dilution (Adderson & Taylor, 1964).

Graphical estimates of the slopes to yield $\partial \ln \text{CMC}/\partial T$ are not accurate. Polynomial expressions relating log CMC and T (the absolute temperature) were therefore obtained using a Stantec Zebra computer. The use of polynomials of higher degrees than those indicated did not give statistically better fits. The experimental and computer-predicted CMC values agree to within 1·2%.

Decyl α-picolinium bromide, log CMC (mol fractions), $= 16{\cdot}37524 - 1{\cdot}750665\ T \times 10^{-1} + 5{\cdot}161737\ T^2 \times 10^{-4} - 4{\cdot}959002\ T^3 \times 10^{-7}$. Dodecyl α-picolinium bromide, log CMC (mol fractions) $= 19{\cdot}57319 - 2{\cdot}104685\ T \times 10^{-1} + 6{\cdot}219035\ T^2 \times 10^{-4} - 5{\cdot}97669\ T^3 \times 10^{-7}$. Tetradecyl α-picolinium bromide, log CMC (mol fractions) $= -1409{\cdot}548 + 21{\cdot}97247\ T - 1{\cdot}367959\ T^2 \times 10^{-1} + 4{\cdot}238423\ T^3 \times 10^{-4} - 6{\cdot}535922\ T^4 \times 10^{-7} + 4{\cdot}014295\ T^5 \times 10^{-10}$.

Table 4. *Thermodynamic quantities for the micellization of alkyl α-picolinium bromides in water*

Temperature °K	$-\Delta G^{\circ}_m$ kcal mol^{-1}	$-\Delta G^{\circ}_m$ kJ mol^{-1}	$-\Delta Hm$ kcal mol^{-1}	$-\Delta Hm$ kJ mol^{-1}	$-\Delta Sm$ cal deg^{-1} mol^{-1}	$-\Delta Sm$ J deg^{-1} mol^{-1}	$-\Delta S^{\circ}_m$ cal deg^{-1} mol^{-1}	$-\Delta S^{\circ}_m$ J deg^{-1} mol^{-1}
Decyl α-picolinium bromide								
278·2	7·78	32·6	—2·1	—8·8	—7·7	—32	—35	—150
283·2	7·95	33·3	—1·5	—6·3	—5·2	—22	—33	—140
288·2	8·11	33·9	—0·84	—3·5	—2·9	—12	—31	—130
293·2	8·26	34·6	—0·22	—0·92	—0·73	—3·1	—29	—120
298·2	8·40	35·2	+0·40	+1·7	+1·3	+5·4	—27	—110
303·2	8·53	35·7	0·99	4·1	3·3	14	—25	—100
308·2	8·65	36·2	1·6	6·7	5·1	21	—23	—96
313·2	8·76	36·7	2·1	8·8	6·7	28	—21	—88
318·2	8·87	37·1	2·6	11	8·1	34	—20	—84
323·2	8·96	37·5	3·0	13	9·4	39	—18	—75
328·2	9·05	37·9	3·5	15	11	46	—17	—71
333·2	9·13	38·2	3·8	16	11	46	—16	—67
338·2	9·21	38·5	4·1	17	12	50	—15	—63
343·2	9·28	38·8	4·3	18	13	54	—14	—59
Dodecyl α-picolinium bromide								
283·2	9·66	40·4	—1·5	—6·3	—5·3	—22	—39	—160
288·2	9·85	40·5	—0·71	—3·0	—2·5	—10	—37	—150
293·2	10·0	41·9	+0·06	+0·25	+0·21	+0·88	—34	—140
298·2	10·2	42·7	0·81	3·4	2·7	11	—31	—130
303·2	10·3	43·1	1·5	6·3	5·1	21	—29	—120
308·2	10·5	43·9	2·2	9·2	7·2	30	—27	—110
313·2	10·6	44·4	2·9	12	9·2	39	—25	—100
318·2	10·7	44·8	3·5	15	11	46	—23	—96
323·2	10·8	45·2	4·0	17	13	54	—21	—88
328·2	10·9	45·6	4·5	19	14	59	—19	—80
333·2	11·0	46·0	5·0	21	15	63	—18	—75
338·2	11·1	46·5	5·3	22	16	67	—17	—71
343·2	11·2	46·9	5·6	23	16	67	—16	—67
Tetradecyl α-picolinium bromide								
283·2	11·2	46·9	0·33	1·4	1·2	5·0	—39	—160
288·2	11·4	47·7	0·32	1·3	1·1	4·6	—39	—160
293·2	11·6	48·5	0·76	3·2	2·6	11	—37	—150
298·2	11·8	49·4	1·5	6·3	5·1	21	—34	—140
303·2	12·0	50·2	2·5	10	8·2	34	—31	—130
308·2	12·1	50·6	3·6	15	12	50	—28	—120
313·2	12·2	51·1	4·6	19	15	63	—24	—100
318·2	12·4	51·9	5·6	23	17	71	—21	—88
323·2	12·5	52·3	6·3	26	20	84	—19	—80
328·2	12·6	52·7	6·9	29	21	88	—17	—71
333·2	12·6	52·7	7·2	30	22	92	—16	—67
338·2	12·7	53·1	7·2	30	21	88	—16	—67
343·2	12·8	53·6	7·1	30	21	88	—17	—71

The change in free energy upon micellization, ΔG°_m, per methylene group is between —0·8 and —0·9 k cal mol^{-1}. These numerical values are similar to those for the free energy change in processes which may be considered analagous, though not identical, namely the transfer of medium chain alcohols (Kinoshita & others, 1958), hydrocarbons (McAuliffe, 1963) or long-chain aliphatic acids (Mukerjee, 1965) from an aqueous to a hydrocarbon environment. This agreement may be fortuitous due to the choice of 2 instead of 2-p/N in the calculation of ΔG°_m.

If the calculated p/N values are used, the free energy change at 25° becomes —0·97 k cal mol^{-1} per CH_2 group on increasing the length from a decyl to dodecyl chain, and —0·77 k cal mol^{-1} per CH_2 for the change dodecyl to tetradecyl chain.

In contrast, Mukerjee (1967) derives ΔG_m^o as -680 cal mol^{-1} per methylene group from the variation in CMC values with alkyl chain length in a homologous series of non-ionic surfactants. The difference between this and the values for complete transfer from aqueous to hydrocarbon environment is attributed to the inadequacy of the liquid core model. He suggests that chains within a micelle are more constrained than in a liquid hydrocarbon and hence make a lower entropic contribution to ΔG_m^o.

The enthalpy and entropy changes are similar to those previously reported (Adderson & Taylor, 1964, 1967) that is that for the shorter chain compounds the enthalpy of micellization is positive at room temperature and becomes negative with temperature increase. For the tetradecyl compound this transition occurs at a lower temperature.

This change in enthalpy from a positive to a negative value with increase in temperature has been recently supported by calorimetric studies of sodium lauryl sulphate (Pilcher & others, 1969).

Benjamin (1964) has reviewed the solution data for aliphatic alcohols in water and derives a value of -590 cal mol^{-1} per methylene group for the enthalpic contribution to the free energy change of solution.

The values of $-0{\cdot}35$ k cal mol^{-1} and $-0{\cdot}20$ k cal mol^{-1} per methylene group at 25° C, which may be derived from the present data for the increments in ΔH_m between decyl and dodecyl and those between dodecyl and tetradecyl salts are thus smaller than those for the above, somewhat analogous, process. The observed lack of uniformity in values of ΔH_m per methylene group is supported by calorimetric data (Benjamin, 1964).

Increase in surfactant hydrocarbon content either by increase in length of alkyl chain or the incorporation of cyclic structures is always observed to be accompanied by increasing exothermicity. Calorimetric determinations of ΔH_m show that increase in total hydrocarbon content converts an endothermic into an exothermic reaction (Corkill & others, 1966), suggesting that the aggregation of head groups is endothermic and that of alkyl chains exothermic.

Micellization is therefore considered to be accompanied by at least three significant enthalpic changes; those associated with aggregation of charged heads, those with breakdown of hydrocarbon-stabilized water structure and changes due to transfer of hydrocarbon from water to micelle. The two former being endothermic, the latter exothermic. The temperature at which ΔH_m changes from a positive to a negative value (at which the CMC shows a minimum value), will vary with the summation of the above. The gradual decrease of hydrocarbon-stabilized water structure with temperature, with consequent decrease in this endothermic component will explain the change of sign of ΔHm. If the electrical work to assemble the monomers is reduced, for instance due to a greater degree of counterion binding in higher homologues, the minimum will occur at a lower temperature. Change of counterion to one more readily bound has the same effect (Adderson & Taylor, 1964; Mukerjee & Ray, 1966). Increase in alkyl chain length with consequent increase in the area of water-hydrocarbon interface lost and the exothermic formation of a larger amount of "liquid hydrocarbon" micelle interior, will depress the temperature of the minimum CMC.

Thus while it must be appreciated that such thermodynamic evidence cannot give

incontrovertible proof, it may be inferred that micellization is entropy-directed at low temperatures but predominantly enthalpy-directed at room temperature and above.

In contrast, Butler & others (1969) consider that ΔHm, derived from the CMC temperature plot, where the CMC is based solely on the concentration of monomers instead of the total surfactant, is always negative and more nearly constant than the values suggested in the present work. Derivation of ΔHm from their CMC values yields values which appear to be further removed from the somewhat scanty calorimetric values for ionic surfactants; this may be a reflection of the imprecise nature of the calorimetric determinations. Similarly ΔGm per CH_2 group is further removed from similar values for transfer of alkyl chains from aqueous to hydrocarbon environment.

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LETTERS TO THE EDITOR

Autacoids in sputum in cases of bronchial asthma and other respiratory diseases

There is much evidence to suggest that pharmacologically active mediators may be involved in production of the asthmatic state. Such mediators were thought likely to be present in sputum, and analysis of sputum might therefore offer direct evidence of their release. The sputum from 79 cases of bronchial asthma during the acute attack and from 44 cases of other respiratory disease was, therefore, examined for the presence of histamine, 5-HT, bradykinin and acetylcholine. The collection of sputum normally had to precede the institution of therapeutic measures, and thus severe cases were excluded from the series. Sputum was collected directly in 10% trichloroacetic acid solution for extraction of histamine and 95% acetone for extraction of 5-HT (Parratt & West, 1957). The specificity of the extraction was further checked by the procedures outlined by Schild (1949). Bradykinin was assayed on rat duodenum and atropinized rat uterus after extraction with a mixture of 95% ethanol in de Jalon solution (Schacter & Morley, 1964; Sardesai, 1968). Sputum was collected in ice cold eserinized Ringer for assay of acetylcholine on frog rectus (Anand, 1952).

Histamine was detected in sputum in 65 cases of bronchial asthma (82·2% of cases) and the amount varied between 0·10–0·49 μg/g of sputum. In 28 cases, examination of the sputum on consecutive days revealed that there was a progressive reduction in the amount of histamine simultaneously with clinical recovery.

The highest amounts of histamine 0·84 $\pm$ 0·354 μg/g were present in sputum of 6 cases of lung abscess. Moderate amounts were present in carcinoid syndrome (0·32 μg/g), in 13 cases of pulmonary tuberculosis (0·26 $\pm$ 0·122 μg/g) and in one case of tropical eosinophilia (0·20 μg/g). Histamine was not detected in patients with bronchiectasis, pneumothorax and congestive cardiac failure.

Apart from a faint trace (2 ng/g) in a patient with lung abscess, 5-HT was not detected in any instance. Bradykinin was present in the sputum in one case of chronic bronchitis and in four out of 6 cases of bronchial asthma; the sputum from other 2 patients contained histamine only. Acetylcholine was not detected in any sample from the 12 cases of bronchial asthma.

Thus there is a direct evidence of the presence of histamine and bradykinin in the respiratory tract in cases of bronchial asthma.

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Effects of oral thymoxamine on blood pressure and pupillary response in normal subjects

Thymoxamine [4-(2-dimethyl-aminoethyl)-5-isopropyl-2-methylphenyl acetate] is a selective adrenergic α-receptor blocking agent. Birmingham, Rubenstein & Latimer (1969) have shown that an intravenous infusion of 0·4 mg/kg to 5 hypertensive patients reclining (3 males and 2 females) produced a statistically significant mean fall of 35 mm Hg in the systolic and 16 mm Hg in the diastolic pressure ($P < 0·001$), but no significant effect in healthy normotensive males.

Myers, Hobbs & Irvine (1968) found that rapid intravenous injection of thymoxamine (10 mg) was accompanied by a transient fall in systemic arterial pressure with an increase in cardiac output and minimal tachycardia. The drug's α-blocking action has been shown in the eye by the antagonism of mydriasis induced by local sympathomimetic amines (Turner & Snedden, 1968).

We have investigated the effect of thymoxamine on the systolic and diastolic blood pressures and pupillary responses in healthy, normotensive individuals (3 males and 3 females, aged 20–36 years) both reclining and standing. Under double blind conditions, oral doses of thymoxamine (50 and 100 mg) and matching placebo in identical capsules were given at weekly intervals in a randomized order based on latin squares half an hour after food on 3 occasions, at the same time of day. One h after administration of the capsules, subjects' pupils were photographed five times at 1 s intervals by a Nikon single lens reflex camera with fixed magnification (Turner & Snedden, 1968). The negatives were projected onto a calibrated white ground glass screen at a magnification of ×8. Pupil diameters were measured and expressed as the transverse diameter to the nearest mm. Blood pressure recordings were taken from subjects on a couch who had rested for 5 min. Each subject, while in this position, had instilled 2 drops of a buffered solution (pH 6·8–7·2) of 5% phenylephrine into the right eye and 2 drops of 2% tyramine into the left eye. Blood pressure was recorded 2 min after the subject stood up. Measurement of blood pressure, and pupil photography were repeated at 15, 30, 45 and 60 min after instillation of the eye drops. The mean value of the five blood pressure readings from each subject was calculated together with the mean percentage mydriasis at 60 min in both pupils after oral thymoxamine treatment. There was no significant difference between treatments in systolic and diastolic pressures in the subjects reclining or in the systolic pressures of the subjects when standing, but a statistically significant fall was observed in the diastolic blood pressure of standing subjects when placebo values were compared with those after thymoxamine 100 mg (mean difference = 6·3 mm Hg, s.e. 2·24, $t = 2·8$, $P < 0·05$).

Resting pupil diameter was not decreased by thymoxamine, unlike antihypertensive drugs such as reserpine and α-methyldopa given by mouth (Snedden & Turner, 1969). A significant reduction in percentage mydriasis was observed in the phenylephrine-treated eye after thymoxamine 50 mg (mean difference 13·01%, s.e. = 4·17, $t = 3·11$, $P < 0·05$).

These results demonstrate that thymoxamine, 100 mg, in capsule form taken after food produces a significant fall in diastolic blood pressure in standing, but not reclining normal subjects. In view of the difference in response to intravenous thymoxamine of hypertensive patients compared with normal subjects (Birmingham & others, 1969), a study of the effects of oral thymoxamine in this formulation in patients with hypertension would seem to be indicated.

Changes in mydriatic response to sympathomimetic amines were small and not comparable with those obtained by local administration of thymoxamine (Turner

& Snedden, 1968). There was a large variation in the changes produced but the significant fall in phenylephrine mydriasis after thymoxamine, 50 mg, compared with the placebo value indicates that sufficient drug or metabolite reached the pupillary tissue to produce some degree of receptor blockade.

We thank the Mental Health Research Fund for providing apparatus used, and William Warner & Sons Ltd. for the capsules of thymoxamine and placebo. A. G. Arbab is supported by a Colombo Plan Scholarship.

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The stability of glyceryl trinitrate tablets

Tablets of glyceryl trinitrate based on mannitol may be expected to retain their potency for at least two years, as long as they are protected from light and stored in cool and dry conditions (British Pharmaceutical Codex, 1968). Nevertheless, it is sometimes implied that mannitol-based tablets deteriorate rapidly, and are of little use in the relief of anginal pain unless used within a year (Laurence, 1966) or even a few months (Mathews & Turck, 1969) of preparation. Any reduction in therapeutic effectiveness will be reflected in the hypotensive response to glyceryl trinitrate (Bernstein, Friesinger & others, 1966; Carson, Wilson & others, 1969). The effectiveness of mannitol-based glyceryl trinitrate tablets has been objectively assessed by measuring the hypotensive response of healthy young medical students to the same batch of tablets over three years.

Glyceryl trinitrate tablets (0·5 mg), prepared with a mannitol base, were stored in a capped brown glass bottle on an open shelf at room temperature. No special precautions were taken to protect the tablets from light. Placebo tablets, identical in appearance but containing only mannitol, were prepared at the same time (in 1967) and were similarly stored.

Each subject was given glyceryl trinitrate and the placebo at different times, and the effect on systolic blood pressure was measured with a sphygmomanometer by auscultation. The difference between the initial blood pressure and the value 6 min after sublingual administration of the drug or placebo was recorded; from these values, the hypotensive effect of glyceryl trinitrate in each subject was calculated.

The results (Table 1) are based on measurements in 133 sitting and standing subjects. In sitting subjects, the mean decrease in blood pressure produced by the drug declined from 10 mm Hg in 1967 to 7 mm Hg in 1969; differences between

these mean values were not statistically significant ($P > 0.05$). A similar response in normal subjects was observed by Besser, Curwen & Duncan (1966), on whose procedure the trial was based. In standing subjects, the mean response was greater (13–15 mm Hg). Throughout, there was a large variation in the hypotensive response in different individuals (Table 1). Approximately 70–80% of subjects complained

Table 1. *The hypotensive effect of glyceryl trinitrate during the three years of the study*

		Reduction in systolic blood pressure induced by 0·5 mg glyceryl trinitrate (mm Hg)					
		Standing			Sitting		
Year	Total number of subjects	Mean ± s.e.	Range	*P*	Mean ± s.e.	Range	*P*
1967	47	14 ± 3	7–22	—	10 ± 1	3–25	—
1968	46	15 ± 1	7–25	0·74	10 ± 2	0–24	0·90
1969	40	13 ± 3	4–21	0·81	7 ± 2	0–21	0·18

P represents the probability that the differences in the hypotensive response between 1967 and the two successive years are due to chance.

of headache of varying intensity, and in 5% of subjects the hypotensive response caused faintness. On chemical analysis two and a half years after their preparation, the tablets contained more than 85% of the stated amount of glyceryl trinitrate, and thus complied with the requirements of the British Pharmacopoeia (1968).

Although measurements were made by relatively inexperienced observers, the results suggest that the tablets retained their potency under ordinary conditions.

The hypotensive response probably reflects the efficacy of these tablets in the relief of ischaemic pain, it is therefore difficult to explain or account for any rapid deterioration in the clinical response to mannitol-based glyceryl trinitrate tablets in patients with coronary ischaemia. Since tolerance to organic nitrates develops and disappears rapidly, an apparent loss of potency might be due to this factor. Alternatively, the rapid deterioration of mannitol-based glyceryl trinitrate tablets may be illusory, and merely reflect the extension of views on chocolate-based tablets, whose limitations are well recognized (Bagnall & Stock, 1955).

My thanks are due to Evans Medical Ltd. for the preparation of the glyceryl trinitrate and placebo tablets. I am also indebted to Mr. G. S. Porter, of the Liverpool Regional College of Technology, for the assay of the tablets.

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An examination of some oil-in-water emulsions by electron microscopy

Various methods have been used to obtain information about the structures responsible for the semi-solid consistency of oil-in-water emulsions containing self-bodying waxes (Axon, 1957; Barry, 1968, 1969; Talman, Davies & Rowan, 1967, 1968; Talman & Rowan, 1968). The mean globule size of products made and analysed as described previously (Talman & others, 1967) was about 1 μm; this approaches the limit of resolution of the optical microscope, which therefore could not be used for detailed examination. We have now examined some typical emulsions by electron microscopy. The preparations selected contained 50% w/w liquid paraffin, 0·5% w/w cetrimide or cetomacrogol 1000 and (A) 10·0% w/w oleyl alcohol, (B) 0·5% w/w cetostearyl alcohol or (C) 7·0% w/w cetostearyl alcohol. Emulsions (A) and (B) were fluids whereas (C) was a cream. The products were diluted with bovine plasma fraction V (Armour) and a drop of the diluted material placed on a carbon film supported by a 200 mesh copper grid 3 mm in diameter. The liquid was evenly dispersed over the carbon film and then as much liquid as possible drained off. The specimens were dried in a dessicator over silica gel and then examined in a Philips EM100B electron microscope.

Nothing was visible in specimens prepared from the fluid emulsions (A) and (B). The fate of the liquid paraffin globules can not be stated with any certainty but several explanations are possible. Using a technique similar to that described above, Groves & Scarlett (1965) observed numerous globules but the carbon film they employed was treated with Formvar before deposition of the sample. It is possible that the untreated carbon film which we used absorbed the oil. Alternatively, the globules may have collapsed during the course of specimen drying to give a thin layer of oil over the carbon surface. A further possibility is that the electron density of the oil was not sufficiently different to that of the carbon film for the former to show up by electron microscopy. Finally the oil may have evaporated at the pressure (about 10^{-5} torr) in the specimen chamber of the electron microscope. At pressures of this order the evaporation rate is independent of the external pressure and the rate was conveniently determined at 10^{-6} torr and at 20° in an apparatus similar to that described by Holland, Laurenson & Deville (1965). The liquid paraffin was contained in a small beaker, its free surface being 3·5 cm below a quartz crystal microbalance cooled to −78° by solid carbon dioxide. As the vapour pressure of ice at this temperature is much greater than that of the system, error due to condensation of the residual water in the apparatus onto the microbalance was avoided. The observed evaporation rate of the liquid paraffin ($2{\cdot}43 \times 10^{-8}$ g cm^{-2} s^{-1}) corresponded to a loss of 1×10^{-7} g of this material per minute from a grid in the specimen chamber of the electron microscope. The amount of the oil deposited on the grid during specimen preparation was estimated to be 10^{-6} to 10^{-5} g and would require 10 to 100 min for complete removal. Furthermore, it is likely that the bovine plasma film used to "fix" the specimen to the grid would retard the evaporation of the oil and also prevent the enhanced evaporation of liquids from droplets as compared with a bulk liquid surface observed by Shapiro & Hanyok (1968). Since globules were not present initially or any time during examination we consider this explanation to be the least likely of the four possibilities.

Figs 1a, b and c were obtained when specimens prepared with emulsion (C) were examined by electron microscopy. The objects had one or more projections which in some cases formed links between them. They did not give an electron diffraction pattern which indicates the absence of an ordered crystal lattice. Shadowing

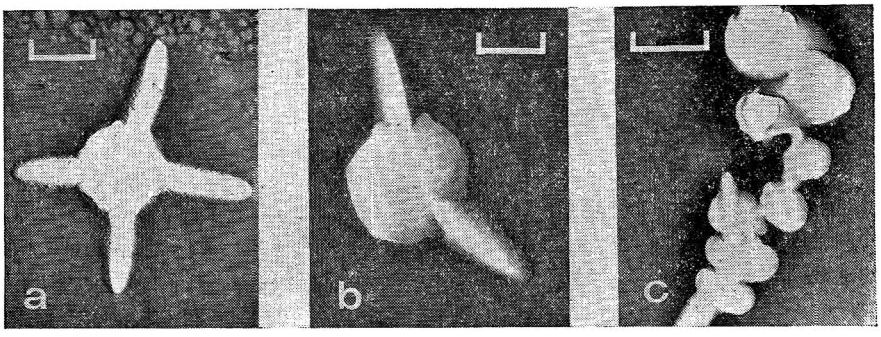

FIG. 1. Electronmicrographs of an emulsion containing 50% w/w liquid paraffin, 7·0% w/w cetostearyl alcohol and 0·5% w/w cetomacrogol 1000. (a) One division = 1 μm, (b) one division = 0·5 μm, (c) one division = 1 μm.

with gold and manganin showed that they were concave and not convex. These objects were examined over a period of hours and the grids were introduced into the specimen chamber more than once and no change was observed. In view of our failure to find emulsion globules when observing specimens prepared from emulsions (A) and (B) we suggest that the objects shown in Figs 1a, b and c are the structuring elements which impart to emulsion (C) its semi-solid consistency. They would be comprised largely of cetostearyl alcohol transferred from the oil to the aqueous phase but it is likely that some water, surfactant and perhaps liquid paraffin may also be present. These materials would "plasticize" the fatty alcohol and allow the structure to collapse when water was removed during the preparation of the specimens, to the shape revealed by shadowing. Fig. 1c is one of a stereoscopic pair which together show a three dimensional network which survived the disruptive effect of the mixing required for specimen preparation.

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A procedure for the estimation of oestradiol, prednisone and testosterone in propylene glycol

Recently the need arose for this laboratory to develop a simple spectrophotometric method for the semi-quantitative estimation of a mixture of oestradiol, prednisone and testosterone in propylene glycol. The major problem was to isolate the testosterone; estimation of the other two ingredients presented no difficulties.

Oestradiol was isolated from a methylene chloride solution of the formulation by extraction into alkali, thorough washing with methylene chloride, acidification and re-extraction into methylene chloride. The solution was washed with water and saturated brine, dried with magnesium sulphate and made up to a known volume. An aliquot was taken, evaporated to dryness and redissolved in an equal volume of ethanol. Prepared mixtures were treated similarly. The absorbance was measured at 280 nm (Scott, 1964a) and compared with a standard solution.

Prednisone was determined by the U.S.P. XVII blue tetrazolium assay for corticosteroids, using an ethanolic solution of the mixture. It was established by means of prepared solutions that the other ingredients did not interfere with the prednisone assay.

Before the testosterone could be estimated, it had to be isolated from the other ingredients, particularly prednisone, since its λ_{max} of 238 nm (MeOH) (The Merck Index, 1968a) was identical with that of the corticoid (The Merck Index, 1968b). The solubility of testosterone in hexane was indicated by Scott (1964b), who reported a value for its absorbance in that solvent, in which prednisone is insoluble.

After removal of oestradiol from the mixture by alkaline extraction, the organic layer was thoroughly washed with water and sodium chloride solution to remove all the propylene glycol. After drying over magnesium sulphate, the solution of testosterone and prednisone in methylene chloride was evaporated to dryness on a rotary film evaporator, and the residue was allowed to form a thin film of glass-like consistency over the inner surface of the flask. A measured volume of hexane was added and the flask was swirled gently for 2–3 min, after which the extract was decanted. This was repeated eight times, with each extract being kept separate. The absorbance of each solution was measured to determine when extraction was complete. The first two extracts had absorbances greater than 2, but the eighth read only 0·03. Thin-layer chromatography [solvent; benzene–methanol (4:1): adsorbent; silica gel G, 0·25 mm] showed only testosterone (Rf = 0·65) in each of the fractions, and only prednisone (Rf = 0·50) in the residue. The extracts were combined in a volumetric flask, after pouring through a glass-wool plug, and the solution was made up to volume. The absorbance was compared with a standard solution. Similar treatment of prepared solutions showed the extraction procedure to be reproducible.

The solvent extraction procedure as described was judged to be superior to all of the TLC separations of the mixture which were attempted in an effort to isolate the testosterone.

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Distribution of tritiated-1-Δ^9-tetrahydrocannabinol in rat tissues after inhalation

Recent controlled studies of marihuana administration to man indicate several unusual pharmacological actions, including a change from mild sedative and euphoric action at low dose, to hallucinogenic effect at high dose; a greater potency when administered by inhalation than by ingestion; and reverse tolerance in that habitual users experience a greater effect per unit dose than naive subjects (Isabell, Gorodetzsky & others, 1967; Hollister, Richards & Gillespie, 1968; Weil, Zinberg & Nelson, 1968).

We have now examined the biological distribution and retention of ^{3}H-Δ-9-tetrahydrocannabinol (Δ^9-THC) administered by inhalation.

^{3}H-Δ^9-THC (13·5 mg, 3370 μCi) (Idänpään-Heikkilä & others, 1969) in light petroleum (30–60°) was injected into a half length cigarette which was then mounted on wire at the bottom of a large vaccuum desiccator. The cigarette was ignited, the desiccator plate was replaced, followed by male Sprague-Dawley rats, 200–250 g, and the desiccator top. After a 15 min exposure to the smoke the animals were transferred into housing cages and decapitated after a 20 min, 8, 24, 72 h, or 7 day interval. All tissues were homogenized in water and 0·1 ml of the homogenate from each tissue in a 10 ml medium containing POP and POPOP was assayed for tritium with a liquid scintillation spectrometer. All samples were corrected for tritiated water, if formed *in vivo* from the ^{3}H-Δ^9-THC, by allowing a duplicate sample to dry in a counting vial before being subjected to liquid scintillation assay. The tissue distribution of ^{3}H-Δ^9-THC in rats at various time intervals is shown in Table 1.

Table 1. *Distribution of radioactivity in rat tissues following inhalation of* ^{3}*H-*Δ^9-THC

Tissue	Ratio of radioactivy (μCi/g) in each tissue to that in brain				
	20 min	8 h	24 h	72 h	7 days
Brain	1·0	1·0	1·0	1·0	1·0
Lungs	8·1	5·3	3·9	1·7	1·0
Liver	1·9	1·4	1·2	1·3	1·0
Kidneys	2·4	1·3	1·2	1·7	0·7
Adrenals	2·1	0·5	0·4	0·3	0·7
Spleen	0·9	0·8	0·7	1·0	0·7
Testis	1·9	0·8	0·8	1·0	1·0
Muscle	2·0	0·3	0·3	0·3	0·7
Fat	1·3	0·5	0·4	0·3	0·7
Salivary Glands	5·8	—*	—*	—*	0·7
Urine†	3·2	3·1	1·3	0·3	0·7
Jejunum	5·6	1·7	1·3	1·3	0·3
Ileum	0·9	3·2	2·0	1·7	0·7
Colon	0·6	4·2	3·8	2·0	1·0

* Samples not taken.
† Sample obtained from the bladder.

The results are expressed as the ratio of radioactivity (μCi/g) in each tissue to that in the brain. As early as 20 min a high accumulation of Δ^9-THC or metabolites, or both, were found in the lungs, salivary glands, jejunum, urine, kidneys, adrenals, muscle, liver, and testis in decreasing order of radioactivity. The extremely high concentration in the lungs was most likely due to the site of administration of the compound. However, the retention of Δ^9-THC in the lungs was obvious, as the radioactivity remained high at 24 h. During the interval between 20 min and 8 h, concentrations in the liver decreased to half, while levels in the brain and kidneys

declined more slowly. Subsequently, tissue concentrations in the brain, liver, and kidneys were maintained constant throughout a period from 8 to 72 h. On the 7th day, an equally high concentration was still found in the brain, lungs, and liver. A high accumulation in the jejunum at 20 min indicated a possible role for biliary excretion and reabsorption of Δ^9-THC or metabolite, or both. Increase of concentrations in the ileum and colon at the later times coincided with the findings of Agurell, Nilsson & others (1969) that the major route of elimination of Δ^9-THC in the body is in the faeces.

The concentrations of radioactivity peaked and fell in all tissues except brain. The decline from the initial brain level at 20 min (0·041 μCi/g) was less than 25% after seven days (0·032 μCi/g). Possibly enough Δ^9-THC or an active metabolite is retained in the brain to account for increased effect per unit dose when smoked habitually.

Unchanged Δ^9-THC (30 to 40% of the total radioactivity), was identified in the lung at 20 min by thin-layer chromatography (TLC) (n-hexane: diethyl ether, 4:1, Silica Gel G) and by a positive colour reaction using *o*-dianisidinetetrazolium chloride spray. Unchanged Δ^9-THC was estimated to be 10 to 20% at 8 and 24 h. The small amount of physical material in the other tissues at all time intervals limited analysis to TLC utilizing a carrier. (Standard Δ^9-THC (red oil) provided by Dr. Sciliagno of the National Institute of Mental Health and synthetic Δ^9-THC provided by Dr. Hines of Hoffman-LaRoche were used as carriers.) Approximately 40% of the radioactivity in the brain at 20 min represented Δ^9-THC; this value had only decreased to 30% at 24 h. In this study, we were unable to further characterize the presence of Δ^9-THC by gas chromatography because of the interference of impurities extracted from the tissues.

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Effects of protriptyline on the depletion of catecholamines induced by 6-hydroxydopamine in the brain of the rat

The compound 6-hydroxydopamine produces a long-lasting depletion of catecholamines when injected intraventricularly into the rat brain. Only catecholamine-containing neurons are affected; brain dopamine being less severely depleted than noradrenaline. This effect appears to be associated with a degeneration of nerve terminals (Bloom, Algeri & others, 1969; Uretsky & Iversen, 1970; Bartholini, Richards & Pletscher, 1970).

The effects of 6-hydroxydopamine and other catecholamine depleting agents on the mouse heart are antagonized by desipramine and protriptyline (Stone, Porter & others, 1964). Desipramine and related drugs are thought to antagonize the peripheral effects of 6-hydroxydopamine by blocking its uptake into sympathetic nerves, which appears to be a prerequisite for the depleting action (Malmfors & Sachs, 1968; Thoenen & Tranzer, 1968). Desipramine and protriptyline are known to be potent competitive inhibitors of the neuronal catecholamine uptake process in sympathetic nerves (Carlsson & Waldeck, 1965; Iversen, 1967; Berti & Shore, 1967).

Desipramine also blocks the uptake of noradrenaline after intraventricular injection of the labelled catecholamine into the rat brain (Glowinski & Axelrod, 1964). Studies of catecholamine uptake in tissue slices from various regions of the rat brain show that noradrenaline uptake is inhibited by desipramine in regions in which noradrenaline neurons predominate, but is less effective in the neostriatum in which dopamine neurons are the most abundant (Häggendal & Hamberger, 1967; Snyder, Green & Hendley, 1968). Fuxe & Ungerstedt (1968), using fluorescence microscopy, found that desipramine and protriptyline inhibited the accumulation of catecholamines by noradrenaline containing neurons but not by dopamine-containing neurons.

In view of these findings it seemed possible that desipramine or protriptyline might selectively antagonize the effects of 6-hydroxydopamine on noradrenaline-containing neurons in the brain, without affecting the actions of the drug on dopamine-containing neurons.

Protriptyline hydrochloride dissolved in saline was administered intraperitoneally (doses expressed as the hydrochloride) to male albino Wistar rats weighing about 160 g. After treatment with protriptyline, 6-hydroxydopamine was given intraventricularly (doses expressed as free base) (Uretsky & Iversen, 1970). All rats were prepared for intraventricular injection (Noble, Wurtman & Axelrod, 1967) on the day before treatment, to facilitate the timing of the protriptyline-6-hydroxydopamine schedule. Animals were killed 4 days after treatment, and their brain removed and weighed before the extraction and fluorimetric assay of dopamine and noradrenaline (Uretsky & Iversen, 1970). Each experiment involved four groups of animals. 1. Saline + artificial csf. 2. Protriptyline + artificial csf. 3. Saline + 6-hydroxydopamine. 4. Protriptyline + 6-hydroxydopamine.

In the first experiment the time course of the effect of protriptyline (10 mg/kg) on 6-hydroxydopamine (250 μg/rat) was investigated, with intervals from 15 to 180 min between administration of the two drugs. Protriptyline indeed antagonized the 6-hydroxydopamine induced depletion of brain noradrenaline at all time intervals (Fig. 1). Brain dopamine, on the other hand, was significantly protected from the actions of 6-hydroxydopamine only at the 60 and 90 min intervals; this may reflect changes in the concentration of protriptyline in the brain with time. Using the optimum interval of 120 min, the effects of 5, 10 and 15 mg of protriptyline/kg on the catecholamine depletion induced by 250 μg of 6-hydroxydopamine were compared (Fig. 2). The optimum dose for protection of noradrenaline was 15 mg of

with protriptyline alone (5–15 mg/kg) were not significantly altered. Overall means for dopamine and noradrenaline levels in control animals were 0·53 ± 0·045 μg/g and 0·34 ± 0·028 μg/g respectively (corrected for recovery).

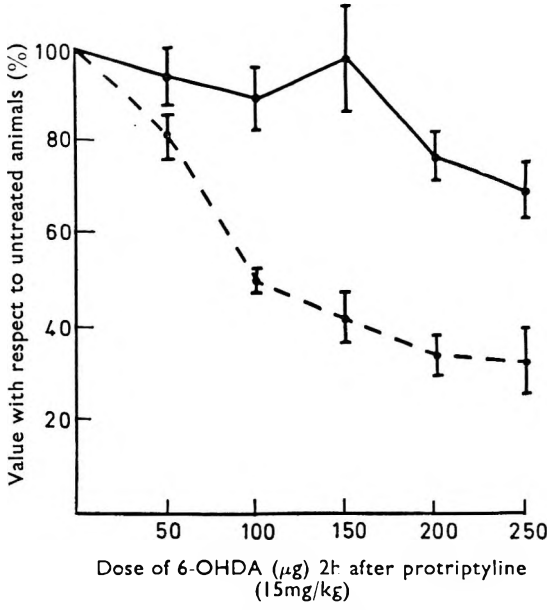

FIG. 3. Depletions of NA and DA induced by various doses of 6-OHDA given 120 min after treatment with protriptyline hydrochloride (15 mg/kg). Details as for Fig. 1.

By pretreatment with protriptyline, it is thus possible to produce animals in which dopamine-containing neurons are chemically lesioned by 6-hydroxydopamine while noradrenaline-containing neurons remain intact. The findings of Coyle & Snyder (1969) indicate that benztropine (cogentin) is a more potent inhibitor of dopamine uptake than of noradrenaline uptake in the rat brain. By pretreatment with benztropine we believe it may be possible to antagonize the effect of 6-hydroxydopamine on dopamine neurons without altering its action on noradrenaline neurons. Studies of the behaviour of animals treated in these various ways are in progress (Evetts, Uretsky & others, 1970).

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Effects of 6-hydroxydopamine on the perfused rat mesentery preparation

We have recently shown in the pithed rat that after pretreatment with 6-hydroxydopamine, an agent producing chemical sympathectomy (Thoenen & Tranzer, 1968), supersensitivity develops to injected noradrenaline (Finch & Leach, 1970). Similar results have been obtained in the spinal cat by Haeusler, Haefely & Thoenen (1969), who observed a 10–30 fold increase in the responses to noradrenaline. In contrast to the cardiovascular responses, the isolated heart exhibited only a 3·5 times greater sensitivity to noradrenaline. It was, therefore, decided to investigate whether 6-hydroxydopamine could produce supersensitivity in the vascular beds.

Male C.S.E. rats, 300–350 g, were given intravenously 6-hydroxydopamine (A. B. Kistner, Gotenborg) (2 × 50 mg/kg on day 1 and 2 × 100 mg/kg on day 7). Perfusion experiments were made on day 10. Mesenteric vessels were isolated and perfused with Krebs solution (McGregor, 1965). Fibres of the periarterial nerve plexus were stimulated at supramaximal voltage (30–40 V), pulse duration 1 ms and a frequency of 6–25 Hz repeated every 2 min. To investigate changes in sensitivity to exogenously administered drugs, noradrenaline and adrenaline (as base) were injected into the perfusate every 3 min in doses of 0·01–1·0 μg.

After pretreatment with 6-hydroxydopamine, the vasoconstrictor responses of the mesenteric preparation to sympathetic nerve stimulation were abolished at low frequencies of stimulation (6, 12 Hz) and markedly reduced at 25 Hz. Noradrenaline sensitivity after treatment with 6-hydroxydopamine was increased by 10–12 times (Fig. 1A). Similar results were obtained for adrenaline. When desipramine (10^{-8} g/ml) was added to the perfusion fluid, no further increase in sensitivity to noradrenaline occurred in the preparations pretreated with 6-hydroxydopamine (Fig. 1B). Control preparations, however, showed potentiated responses similar in extent to those seen in 6-hydroxydopamine-treated preparations.

These results suggest that 6-hydroxydopamine produces chemical sympathectomy of the mesenteric blood vessels. Supersensitivity to injected noradrenaline also occurred and there was no further potentiation after desipramine pretreatment. It would seem, therefore, that chemical sympathectomy abolishes the normal physiological uptake process by day 10 and produces a pre-junctional supersensitivity to noradrenaline. These results are only in partial agreement with those reported by McGregor & Phelan (1969) who found that 6-hydroxydopamine abolished the vasoconstrictor responses to nerve stimulation but did not alter the noradrenaline sensitivity of the perfused mesenteric arteries. Their treatment with 6-hydroxydopamine (30 mg/kg, i.p.) produces only an incomplete depletion of endogenous catecholamines

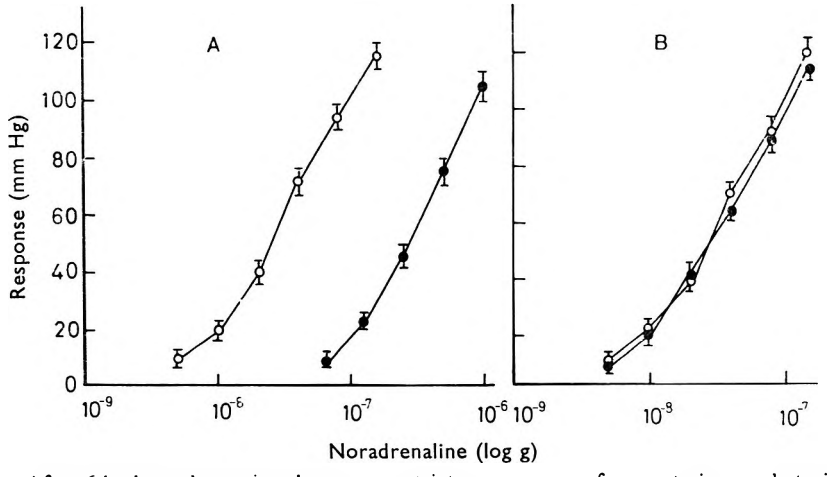

FIG. 1. After 6-hydroxydopamine the vasoconstrictor responses of mesenteric vessels to injected noradrenaline was increased. Each point represents the mean value (± s.e. as vertical bars). (A) ● – ● control responses (6), and ○ – ○ ten days after the first injection of 6-hydroxydopamine (4). (B) Effect of desipramine (10^{-8} g/ml) in the perfusion fluid.[8] ● – ● control responses (6), and ○ – ○ ten days after the first injection of 6-hydroxydopamine (4). Figures in brackets in the legend indicate the number of individual observations.

(Laverty & Phelan, 1969) and there is evidence that the doses we used are more effective in destroying sympathetic nerve endings (Thoenen & Tranzer, 1968; Clarke & Jones, 1969; Finch & Leach, 1970).

In conclusion our results suggest that the increased cardiovascular reactivity to catecholamines, after treatment with 6-hydroxydopamine is at least partially due to supersensitivity of the vascular beds.

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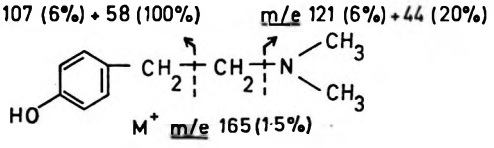
Identity of peyocactin, an antibiotic from peyote (*Lophophora williamsii*), and hordenine

A variety of medicinal uses have been claimed (LaBarre, 1960; Schultes, 1940) for the well-known hallucinogenic peyote cactus, *Lophophora williamsii* (Lemaire) Coulter. McCleary, Sypherd, & Walkington (1960) recently isolated peyocactin, a water-soluble crystalline substance, from an ethanol extract of peyote and found it to be inhibitory *in vitro* against 18 strains of penicillin-resistant *Staphylococcus aureus* and effective in mice against fatal staphylococcal infection. Since there appeared to be no report in the literature elucidating the structure of peyocactin, it became an objective to characterize this antibiotic substance.

The crystalline peyocactin, mp 117–118°, isolated according to the procedure of McCleary & others (1960) and recrystallized thrice from water, was found to be homogeneous by silica gel G thin-layer chromatography in 5 solvent systems (Kirchner, 1967). Diazotized sulphanilic acid (Stahl, 1965) was utilized as the chromogen which also established the phenolic nature of peyocactin. The proton magnetic resonance spectrum of peyocactin in deutero-methanol (tetramethylsilane as internal standard) showed the following signals: δ 2·87 (6H, singlet, $-N\langle^{CH_3}_{CH_3}\rangle$, 3·02 and 3·20



(2H each, multiplets, A_2B_2, $ArCH_2CH_2N$), 6·75 and 7·13 ppm (2H each, doublets, $J = 7$ Hz, AA′BB′, *p*-disubstituted aromatic). The mass spectrum of peyocactin exhibited the molecular ion at *m/e* 165 with major fragments indicated in Scheme 1. These two spectra clearly identified peyocactin as *NN*-dimethyl-*p*-hydroxyphenethylamine (hordenine), which was confirmed by comparison of the spectra with those of an authentic sample of hordenine. Further, the infrared spectra (KBr pellets) of peyocactin sulphate and hordenine sulphate were superimposable and their m.p. (197–198°) was unaltered upon admixture. Similarly the picrate derivatives of the two substances were identical (mp 139–140°).

Hordenine has been previously reported in the peyote cactus (McLaughlin & Paul, 1966; Todd, 1969) and it is also known in the literature as anhaline (*Anhalonium fissuratum*) (Reti, 1953). Earlier studies by Camus (1906) have shown hordenine to be slightly antiseptic. Although McLaughlin & Paul (1966) made reference to this antiseptic action of hordenine in an attempt to account for the bacteriostatic activity of peyocactin, no efforts were made to characterize the antiobiotic. It has now been established that peyocactin is indeed hordenine.

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Antagonism by amantadine of prochlorpemazine-induced catalepsy

Amantadine (1-adamantanamine hydrochloride), an antiviral agent (Davies, Grunert & others, 1964) was given by Schwab, England & others (1969) to prevent influenza in a patient with Parkinson's disease with a remarkable effect on the parkinsonian symptoms. These authors confirmed on 163 patients with Parkinson's disease the efficacy of amantadine "usually consisting of a reduction of akinesia and rigidity and some lessening of tremor". These results were recently confirmed by a controlled trial of Parkes, Zilkha & others (1970).

The toxicological and pharmacological properties of amantadine were examined by Vernier, Harmon & others (1969) who observed, at high doses, a moderate increase of spontaneous motor activity in mice, an antagonism of tetrabenazine-induced sedation in mice and some convulsions at toxic doses.

We studied some effects of amantadine in rats and mice (after intraperitoneal administration, doses are expressed in weight of amantadine). The excitation was moderate between 4 and 64 mg/kg; at these doses, hypothermia and mydriasis were noticed. At doses between 1 and 64 mg/kg, we did not observe any antagonism against oxotremorine-induced hypothermia and tremors or against reserpine-induced hypothermia and ptosis in mice. Amantadine, at doses higher than 16 mg/kg, antagonized the loss of righting-reflex induced by pentobarbitone or barbitone in mice. When administered 30 min before (+)-amphetamine sulphate (3 mg/kg, i.p. in rats), amantadine (at doses higher than 4 mg/kg) significantly increased the intensity of stereotypicities (licking, gnawing . . .); this effect was less marked if amantadine was injected 1 h before (+)-amphetamine and non-existent if the interval was 2 h.

Considering the important therapeutic effect of amantadine on akinesia and rigidity, we examined the effect of this drug on the prochlorpemazine-induced catalepsy in rats.

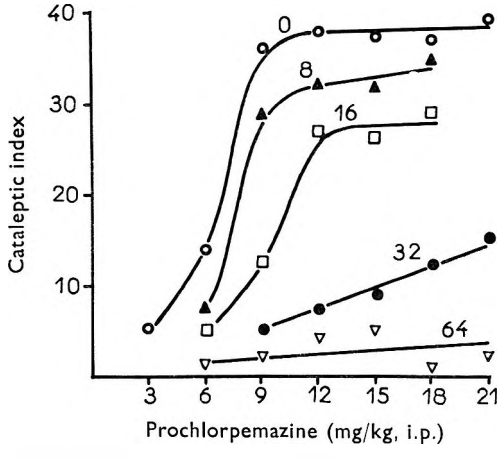

FIG. 1. Homolateral legs crossing test. The "cataleptic index" was calculated by adding the number of cataleptic rats, the homolateral legs crossing test being performed every hour during 7 h (6 animals per group, maximal cataleptic index = 42). The numbers on the curves indicate the dose of amantadine previously administered.

Table 1. *Anticataleptic effect of amantadine.* The CD50 (cataleptic dose) of prochlorpemazine is an arbitrary measurement calculated graphically. It represents the dose of prochlorpemazine inducing catalepsy in 50% of the rats during the 7 h of the test (catalepsy was estimated every hour and the results obtained at each hour were added)

Pretreatment	CD 50 prochlorpemazine mg/kg., i.p. 3 cm-high cork	9 cm-high cork	Hom. legs crossing	Parallel bars	Grid
—	5	6·5	7	7	7
Amantadine 8 mg/kg	6·5	8	8	9	8
" 16 "	9	13	11	17	14
" 32 "	13	21	>21	>21	17
" 64 "	>21	>21	>21	>21	>21

We used four tests (3 cm high-cork, 9 cm high-cork, crossing of homolateral legs, parallel bars) previously detailed by Simon, Langwinski & Boissier (1969) and the grid test. The rat was placed gently on a vertical grid; it was considered as cataleptic if it did not move its paws during 20 s. These five tests were made every hour during 7 h after prochlorpemazine treatment (3, 6, 9, 12, 15, 18 or 21 mg/kg, i.p.); amantadine (0, 8, 16, 32 or 64 mg/kg, i.p.) was injected 15 min before prochlorpemazine (groups of 6 rats). The results obtained with the homolateral legs crossing tests are in Fig. 1. The catalepsy-inducing doses (CD 50) of prochlorpemazine on the five different tests after administration of various doses of amantadine are in Table 1.

The clear antagonism of amantadine towards prochlorpemazine-induced catalepsy could have allowed one to predict the efficiency of this drug in patients with Parkinson's disease (Boissier & Simon, 1964). Besides, the stimulating effects of amantadine, as shown by the observation of the behaviour of animals, by the potentiation of (+)-amphetamine and by the antagonism to barbiturates, corroborate some of the side-effects (jitteriness, insomnia, dizziness) described in patients by Schwab & others (1969).

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Central dopamine and noradrenaline receptor activity of the amines formed from *m*-tyrosine, α-methyl-*m*-tyrosine and α-methyldopa

Like the dopamine and noradrenaline precursor, L-3,4-dihydroxyphenylalanine, the amino-acids *m*-tyrosine, α-methyl-*m*-tryrosine and α-methyldopa are decarboxylated *in vivo* to amines, which are partly β-hydroxylated in the noradrenaline neurons (Carlsson & Lindqvist, 1962; 1967). Treatment with α-methyldopa but not with α-methyl-*m*-tyrosine produces hypotension in rats (Henning, 1967). The hypotensive effect of α-methyldopa appears to be due to formation of central amines since it is not observed after inhibition of the central decarboxylase activity (Davis, Drain & others, 1963) but is present after selective inhibition of the peripheral decarboxylase activity (Henning, 1969). Other pharmacological effects may also be induced by the amines formed in the central nervous system. Very little is known, however, about the central receptor activity of these dopamine and noradrenaline analogues. Therefore we have studied a possible stimulation of the dopamine receptors in the corpus striatum and of the noradrenaline receptors in the spinal cord after treatment with these amino-acids. To exclude an indirect effect of the amines formed, the animals were always pretreated with a large dose of reserpine (10 mg/kg i.p. 10–18 h before the amino-acids) and often also with the tyrosine hydroxylase inhibitor DL-α-methyltyrosine methylester HCl (H 44/68; 250 mg/kg i.p., 1–2 h before). The amines formed from *m*-tyrosine are, in contrast to the α-methylated amines, to a large extent oxidatively deaminated. Therefore, the monoamine oxidase inhibitor nialamide (50 mg/kg, i.p.) was given 1 h before injection of *m*-tyrosine but not before the other amino-acids.

The functional effect of the amine metabolites on the dopamine receptors in the corpus striatum was examined after unilateral removal of the corpus striatum of adult, male Sprague-Dawley rats (Andén, Dahlström & others, 1966). Stimulation of the noradrenaline receptors by the amine metabolites was tested in acutely spinalized rats by pinching the hindlegs and evaluating the strength of the flexor reflex activity (Andén, Corrodi & others, 1967). The locomotor activity of rats was recorded in Animex boxes (Svensson & Thieme, 1969).

The amines were purified and separated by cation exchange chromatography and determined spectrofluorimetrically after condensation with *o*-phthalaldehyde (*m*-tyramine, *m*-octopamine, α-methyl-*m*-tyramine, metaraminol; Shore & Alpers, 1964) or

Table 1. *Concentrations (μg/g) of dopamine and noradrenaline analogues in the rat brain and spinal cord at various times after injection of their precursor amino-acids. The rats were pretreated with reserpine* (10 mg/kg, i.p., 10–18 h *before*). *Means ± s.e. of* 3 *experiments*

Precursor	Amine	Mean recovery %	Brain (B) and spinal cord (S) 0 h B	0 h S	½ h B	½ h S	1½ h B	1½ h S	6 h B	6 h S
DL-*m*-Tyrosine (150 mg/kg, i.p., 1 h after nialamide 50 mg/kg, i.p.)	*m*-Tyramine	65	0·04 ±0·01	0·07 ±0·00	13·30 ±1·70	13·22 ±1·21	3·51 ±0·69	3·06 ±0·40	0·11 ±0·01	0·19 ±0·05
	m-Octopamine	81	0·01 ±0·00	0·01 ±0·00	0·15 ±0·01	0·15 ±0·01	0·17 ±0·02	0·15 ±0·09	0·07 ±0·01	0·08 ±0·05
DL-α-Methyl-*m*-tyrosine (800 mg/kg, i.p.)	α-Methyl-*m*-tyramine	91	0·03 ±0·00	0·04 ±0·01	—		0·55 ±0·11	0·21 ±0·03	0·37 ±0·04	0·25 ±0·01
	Metaraminol	80	0·03 ±0·00	0·02 ±0·00	—		0·19 ±0·03	0·15 ±0·02	0·33 ±0·06	0·26 ±0·04
L-α-Methyldopa (400 mg/kg, i.p.)	α-Methyldopamine	91	0·00 ±0·00	0·00 ±0·00	0·20 ±0·00	0·13 ±0·02	0·27 ±0·06	0·07 ±0·02	0·38 ±0·05	0·12 ±0·01
	α-Methylnoradrenaline	87	0·02 ±0·01	0·02 ±0·00	0·06 ±0·00	0·04 ±0·01	0·14 ±0·01	0·08 ±0·02	0·21 ±0·02	0·12 ±0·04

after oxidation (α-methyldopamine; Carlsson & Lindqvist, 1962) or after boiling and oxidation (α-methylnoradrenaline; Waldeck, 1968). In each experiment, the pooled brains and the pooled spinal cords of 4 rats were analysed. The brain extract was divided in two aliquots and a known amount of amines was added to one of them. All values were corrected for the mean recovery (see Table 1).

Dopamine receptor activity. Injection of DL-*m*-tyrosine (150 mg/kg, i.p.) to unilaterally striatotomized rats pretreated with reserpine and nialamide caused the rats to turn the head and tail from the unoperated to the operated side. This effect was noted after approximately 15 min, was maximal after 30–60 min and had almost disappeared after 2 h. At the peak of action, the rats were active and rotated to the operated side. Injection of L-α-methyldopa (400 mg/kg, i.p.) usually produced a decrease in the reserpine-induced turning but never caused a deviation to the operated side. No change in the reserpine-induced turning was observed after injection of DL-α-methyl-*m*-tyrosine (800 mg/kg, i.p.). The effects mentioned were also seen after pretreatment with reserpine plus H 44/68, thus excluding an amphetamine-like action (see Andén, Carlsson & Häggendal, 1969).

Noradrenaline receptor stimulation. Injection of DL-*m*-tyrosine in doses up to 800 mg/kg, i.p., did not cause any marked increase in the flexor reflex activity of spinal rats pretreated with reserpine and nialamide. Treatment with DL-α-methyl-*m*-tyrosine (800 mg/kg, i.p.) was also ineffective. On the other hand, there was a pronounced increase in flexor reflex activity after administration of reserpine plus L-α-methyldopa (400 mg/kg, i.p.). This effect began after about $1\frac{1}{2}$ h, was maximal after 4–8 h and was still observed, though weaker, the following day. Pretreatment with H 44/68 had no effect on this increased flexor reflex activity. However, administration of the adrenergic α-receptor blocking agent phenoxybenzamine (20 mg/kg, i.p., 4 h after α-methyldopa) or the dopamine-β-hydroxylase inhibitor bis(4-methyl-1-homopiperazinylthiocarbonyl)disulphide (FLA-63 25 mg/kg, i.p. 30 min before α-methyldopa) did block this effect. The hypotensive effect of α-methyldopa is abolished by FLA-63 (Henning & Rubenson, unpublished observations).

Behaviour. The injection of DL-*m*-tyrosine (75–150 mg/kg, i.p.) to reserpine- and nialamide-treated rats induced a marked increase in motor activity to even above normal values in agreement with the findings in mice described by Blaschko & Chruściel (1960). The onset and duration of these effects corresponded to the dopamine receptor stimulation described above. Particularly after more than 30 min, the hyperkinesia was accompanied by stereotypies such as sniffing, chewing of woodshavings and backward walking. The effects were observed also after pretreatment with H 44/68. Injection of L-α-methyldopa (400 mg/kg, i.p.) somewhat increased the motor activity of the reserpine–H 44/68-treated rats although not as markedly as described by Uretsky & Seiden (1969). No behavioural changes were seen after administration of DL-α-methyl-*m*-tyrosine (800 mg/kg, i.p.).

Chemistry. The concentrations of the amine metabolites formed from the various amino-acids in the brain and the spinal cord are presented in Table 1. After injection of DL-*m*-tyrosine there was a rapid and marked accumulation of *m*-tyramine and a smaller accumulation of its β-hydroxylated derivative metaoctopamine. Both amines had almost disappeared after 6 h. After injection of DL-α-methyl-*m*-tyrosine or L-α-methyldopa, the peak concentrations of the corresponding amines were reached later and were much lower for the non-β-hydroxylated amines (α-methyl-*m*-tyramine, α-methyldopamine) than after *m*-tyrosine. The amine levels were of about the same magnitude after the two α-methylated amino-acids. No significant concentrations of dopamine, noradrenaline and the amines described above were observed after treatment with reserpine alone (0 h in Table 1). Treatment with FLA-63 (25 mg/kg,

i.p.) 30 min before L-α-methyldopa (400 mg/kg, i.p., 6 h, n = 3) did not significantly change the level of α-methyldopamine (0·54 ± 0·086 μg/g in the brain, 0·11 ± 0·024 μg/g in the spinal cord) but markedly reduced that of α-methylnoradrenaline (0·06 ± 0·090 μg/g in the brain, 0·01 ± 0·001 μg/g in the spinal cord).

From this functional and chemical evidence it appears that there is a correlation in time of the functional effects of *m*-tyrosine and α-methyldopa and the peak accumulation of *m*-tyramine and α-methylnoradrenaline, respectively. There was a much greater and faster accumulation of *m*-tyramine than of the other amines which may be of importance for the dopamine receptor stimulation. All the β-hydroxylated amines reached about the same peak concentrations but stimulation of the noradrenaline receptors was only seen after injection of α-methyldopa.

In conclusion, treatment with *m*-tyrosine and α-methyldopa caused a stimulation of central dopamine and noradrenaline receptors, respectively, whereas no effect on these receptors was observed after treatment with α-methyl-*m*-tyrosine.

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Effects of chronic morphine administration on the catecholamine depletion induced by reserpine

Morphine interferes with the depletion of brain noradrenaline seen after reserpine in acute (Freedman, Fram & Giarman, 1961) and chronic experiments (Gunne, 1963). After chronic administration of morphine there was only a 38% reduction of brain noradrenaline 20 h after an injection of reserpine compared with a 93% reduction in control animals without morphine.

To establish the cellular localization of these effects, especially those of the various

catecholamine terminals and cell body systems in the central nervous system (Fuxe, 1965), a histochemical catecholamine analysis (see Falck, Hillarp & others, 1962) has also been made.

Male white Sprague-Dawley rats, 250 g, had morphine HCl intraperitoneally in increasing doses up to 180 mg/kg twice daily for three weeks (for details, see Gunne, 1963). A single injection of reserpine (5 mg/kg, i.p.) was then given 4 h after the morning dose of morphine. The animals were divided into 3 groups. (i) Control group, given reserpine and killed at the same time intervals as the morphine-treated rats (4, 12, 20, 28, 48, 72 h after reserpine). (ii) Morphine-tolerant group, given morphine (180 mg/kg, every 8 h) after the reserpine. (iii) Withdrawal group not given morphine after the reserpine.

Brain and adrenal catecholamine levels were measured (Euler & Lishajko, 1961; Carlsson & Lindqvist, 1962). Animals (2 or 3) were taken for histochemistry from each experimental group. The tel- and diencephalon, mesencephalon, pons and the medulla oblongata were first reacted with formaldehyde gas, generated from paraformaldehyde stored at 70% relative humidity; and then reacted with formaldehyde gas generated from paraformaldehyde stored at 90% relative humidity (see Dahlström & Fuxe, 1964; Fuxe & Jonsson, 1967).

In the controls there was a rapid and profound depletion of noradrenaline to 11% of the starting concentration and this depletion lasted longer than in the morphine-treated groups (Fig. 1A). At 72 h after the reserpine injection the noradrenaline concentration was still 26% of normal in the controls. Continuous administration of morphine reduced the degree of depletion. The noradrenaline was 59% at 12 h after reserpine and 48% at 48 and 72 h. In the withdrawal experiment, the noradrenaline concentrations were also higher than in the controls, the mean varying between 45% at 28 h and 75% at 48 h after reserpine.

A corresponding pattern was obtained in brain dopamine (Fig. 1B), a depletion occurring in the controls during the first 28 h with tissue concentrations down to 0·5% of initial values and only an incomplete recovery at 72 h (40%). In the morphine-tolerant animals the depletion was only moderate, the tissue concentrations remaining between 43 and 75% of the starting value. The dopamine concentrations in the withdrawal group returned to normal sooner than did noradrenaline, and in both instances the 48 h amine level differed from the morphine-tolerant rats ($P<0{\cdot}05$).

The histochemical studies showed a catecholamine depletion after reserpine within cell bodies and terminals only in the control rats. At 20–28 h after reserpine, a recovery of fluorescence was seen in all catecholamine cell bodies and at the end of the experiment a clearcut recovery of fluorescence had occurred in the catecholamine nerve terminals (see Dahlström & Fuxe, 1964). In the morphine-treated animals, on the other hand, there was no noticeable reduction of fluorescence within the noradrenergic or dopaminergic neuron cell bodies. Only in the nerve terminals was there a moderately reduced degree of fluorescence.

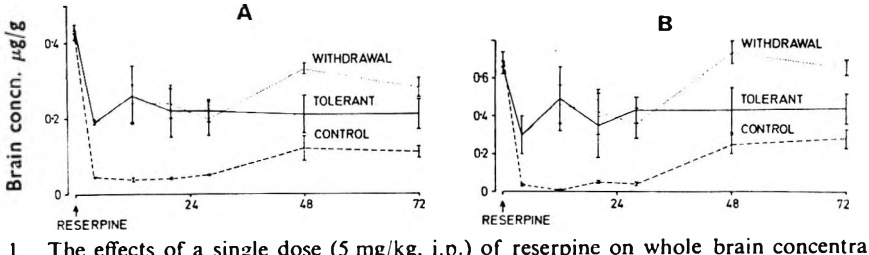

FIG. 1. The effects of a single dose (5 mg/kg, i.p.) of reserpine on whole brain concentrations of A, noradrenaline B, dopamine, at various time intervals after reserpine injection. Broken line = control group; solid line = morphine tolerant group; dotted line = withdrawal group.

The observation (Gunne, 1963) that the catecholamine-depleting action of reserpine in the brain is partially blocked by chronic morphine administration was confirmed. Furthermore, this antagonism of reserpine lasted the three day experiment, even when morphine was withheld. In the latter case the brain dopamine and noradrenaline nerve terminals had reached normal or nearly normal values within 48 h after reserpine at a time when the amine values in the controls were still low. The mechanism of this antagonism between morphine and reserpine is unknown. It has been shown, however, that the reserpine-induced catecholamine depletion can be partially reversed by pretreatment with other substances besides morphine, such as *m*-tyrosine and tetrabenazine (Quinn, Shore & Brodie, 1959; Carlsson & Lindqvist, 1967). In these cases the protection is usually believed to be the result of a competition for the uptake-storage mechanism by reserpine and the drug used for pretreatment. A similar mechanism may exist with morphine.

In the morphine withdrawal group there was a large replenishment of dopamine and noradrenaline stores 48 and 72 h after resperine. In the morphine tolerant group, the brain catecholamine remained at about 50% during the 3 day experiment, probably due to an iterated morphine-induced liberation of catecholamine (Gunne, Jonsson & Fuxe, 1969).

There was no evidence of a release of brain catecholamine from withdrawal stress in these rats. Such a depletion as a result of morphine withdrawal has been reported in dogs, but is an irregular finding in rats (Gunne, 1963). The reserpine syndrome was less severe in the morphine tolerant rats. There was less reduction in exploratory behaviour, less pronounced hunched back posture and less decrease in response to various stimuli than in the reserpine-treated control rats. It seems likely that the rapid recovery from the effects of reserpine on gross behaviour in the morphine-treated rats seen in this study is related to the incomplete depletion of brain dopamine and noradrenaline nerve terminals.

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