Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

Volume 20 No. 10
October 1968

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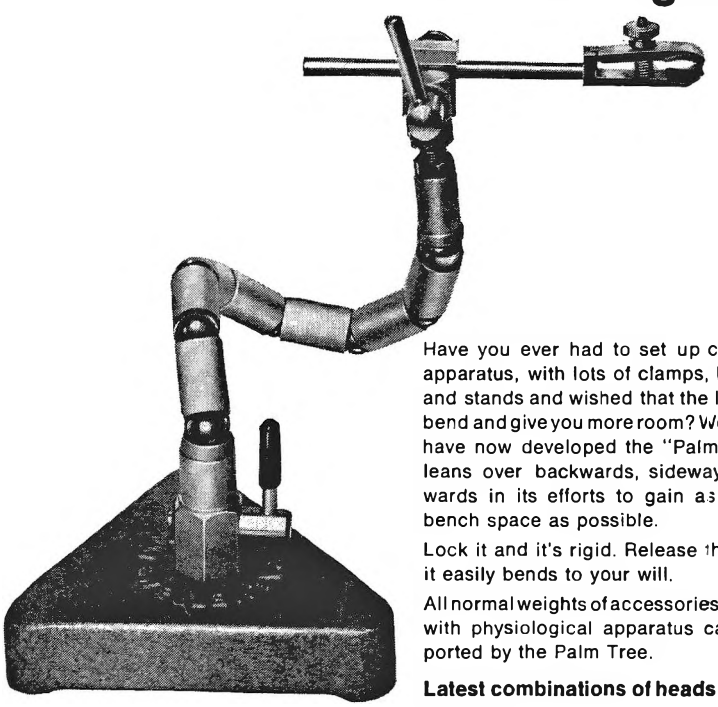

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J. Pharm. Pharmac., 1968, **20**, 737–743 Received April 24, 1968

Influence of ω-amino-acids on blood pressure, catecholamine stores and the pressor response to physostigmine in the rat

P. R. HEDWALL, L. MAÎTRE AND H. BRUNNER

The effects of aliphatic ω-amino-acids of various chain lengths, i.e. glycine, γ-aminobutyric acid, δ-amino-valeric acid, ε-aminocaproic acid and 7-amino-oenanthic acid, on blood pressure and myocardial catecholamine stores in the renal hypertensive rat, and the pressor response to physostigmine in the normotensive rat were studied. The effects of the ω-amino-acids were compared with those of guanethidine and α-methyldopa. ε-Aminocaproic acid, like guanethidine and α-methyldopa, produced a fall in blood pressure, a decrease in catecholamine stores and inhibited the pressor response to physostigmine. GABA reduced blood pressure, but did not affect the other parameters. δ-Amino-valeric acid reduced blood pressure and depleted catecholamine stores slightly, but did not inhibit the pressor response to physostigmine. The other ω-amino-acids were inactive.

EPSILON aminocaproic acid (EACA) is currently in clinical use as an antifibrinolytic agent. Depletion of myocardial noradrenaline stores in the rat and mouse after treatment with EACA has been described by Lippmann, Wishnick & Buyske (1965), and this effect has been related to inhibition of the dual amine uptake concentration mechanism of the adrenergic neurons (Obianwu, 1967a). In addition, EACA has been reported to produce adrenergic nerve blockade (Andén, Henning & Obianwu, 1968).

Another ω-amino-acid, γ-aminobutyric acid (GABA), has been reported to have an antihypertensive effect in man (Takahashi, Tiba & others, 1956), which is attributed to an influence on the central nervous system rather than to an effect on peripheral sympathetic transmission (Belloni, Savioli & Barbieri, 1966).

The present paper describes the effects of ω-amino-acids of various chain lengths on blood pressure and catecholamine stores in the renal hypertensive rat. In addition, the effect of these compounds on the pressor response to noradrenaline and physostigmine was studied in the normotensive rat. The effects are compared with those of two antihypertensive agents which are known to cause a depletion of noradrenaline from its catecholamine storage site, i.e. guanethidine and α-methyldopa.

Experimental

METHODS

Antihypertensive effects in renal hypertensive rats. Male rats were made hypertensive by constriction of the left renal artery. After stable near-systolic blood pressure levels of 170 mm Hg and above had been reached, the animals were divided into groups of 6–23 rats each with similar mean blood pressure levels. Blood pressure was measured at the beginning of the treatment period, 2 and 24 hr after the first administration, 24 hr

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after the second administration, 2 and 24 hr after the third administration and 2 hr after the fourth administration. The plethysmographic method of Byrom & Wilson (1938) was used.

The animals were treated daily for 4 days with the following compounds and doses: guanethidine, 3 mg/kg/day subcutaneously; DL-α-methyldopa (α-methyldopa), 300 mg/kg/day orally; glycine, 1000 mg/kg/day intraperitoneally; GABA, 300 mg/kg/day orally, 1000 mg/kg/day intraperitoneally; δ-aminovaleric acid (DAVA), 1000 mg/kg/day intraperitoneally; EACA, doses as for GABA; 7-amino-oenanthic acid (7-AOA), 1000 mg/kg/day intraperitoneally.

The effect of a single intraperitoneal dose of 1000 mg/kg EACA on blood pressure was followed for 14 hr after administration. In parallel groups of 13 hypertensive rats each, blood pressure was measured 2, 4, 6, 8 and 14 hr after administration of EACA or 0·9% saline.

Noradrenaline concentrations in heart and brain of renal hypertensive and normotensive rats. Twenty four hr after the fourth administration of α-methyldopa or 2–2½ hr after the fourth administration of the other compounds, the animals were bled and the organs removed. The noradrenaline of pooled tissue homogenates of two animals was extracted twice with 10% trichloroacetic acid, adsorbed onto alumina at pH 8·4, eluted with 0·25N HCl and measured fluorometrically (Euler & Orwén, 1955; Euler & Lishajko, 1959).

In a separate experiment, the effect of a single intraperitoneal dose of 1000 mg/kg EACA on myocardial catecholamine concentrations in normotensive rats was determined at various intervals until catecholamine levels returned to normal.

Pressor responses to noradrenaline and physostigmine. Normotensive male rats were treated daily for 4 days. Guanethidine (3 mg/kg/day) was injected subcutaneously and α-methyldopa (300 mg/kg/day) was given orally. Glycine, GABA, DAVA, EACA and 7-AOA (1000 mg/kg/day) were administered intraperitoneally. Two hr after the fourth administration the animals were anaesthetized with urethane (1·7 g/kg, intraperitoneally) and blood pressure in the carotoid artery was recorded with a mercury manometer. Noradrenaline (6 μg/kg) or physostigmine salicylate (150 μg/kg) were injected into a canulated jugular vein. The maximal increases in blood pressure produced by noradrenaline or by physostigmine in parallel groups of treated and untreated control animals were compared. For details of methods above, see Brunner, Hedwall, & others (1967). α-Methyldopa was administered as a suspension in acacia; all other substances were dissolved in 0·9% saline solution. The statistical methods of Lord (1947) and Hogben (1964) were used.

Results

Antihypertensive effects in renal hypertensive rats. The ω-amino-acids, GABA and EACA, produced no significant change in blood pressure after oral administration (Table 1). After intraperitoneal administration of 1000 mg/kg, GABA, DAVA and EACA reduced blood pressure to approximately the same extent as 300 mg/kg/day α-methyldopa given orally, while

TABLE 1. ANTIHYPERTENSIVE EFFECTS IN RENAL HYPERTENSIVE RATS

Treatment Initial blood pressure value mm Hg (n)	Change in blood pressure in mm Hg					
	2 hr after 1st admin.	24 hr after 1st admin.	24 hr after 2nd admin.	2 hr after 3rd admin.	24 hr after 3rd admin.	2 hr after 4th admin.
0·9% NaCl 2 ml/kg/day oral 209±4 (14)	−5±5	−15±5	−15±5	−19±4	−19±5	−26±4
2 ml/kg/day i.p. 213±3 (16)	−6±4	−14±3 (n=10)	−12±5 (n=10)	−10±3	−8±4	−21±5
Guanethidine 3 mg/kg/day s.c. 188±2 (12)	−34±6***	−38±4***	−48±4***	−70±4***	−52±4***	−69±4***
α-Methyldopa 300 mg/kg/day oral 188±3 (23)	−20±3**	−25±6	−29±5*	−40±4***	−34±4***	−39±4*
Glycine 1000 mg/kg/day i.p. 202±4 (6)	−22±3*	−11±4	−7±2	−23±2	−12±3	−24±6
GABA 300 mg/kg/day oral 205±5 (7)	−16±6	+4±5	+6±5	−16±5	+13±5	−11±5
1000 mg/kg/day i.p. 205±7 (7)	−54±6***	+6±4	0±3	−43±6***	−6±7	−42±8*
DAVA 1000 mg/kg/day i.p. 213±4 (6)	−39±6***	0±16	−16±9	−53±5***	−12±8	−34±6
EACA 300 mg/kg/day oral 205±4 (7)	−6±3	−4±4	+5±5	−8±6	−7±6	−11±7
1000 mg/kg/day i.p. 206±3 (13)	−18±4	−8±3	−1±4	−36±5***	−2±6	−42±5*
7-AOA 1000 mg/kg/day i.p. 202±7 (6)	−8±3	−8±6	−8±5	−25±6	−15±2	−24±3

Values are means ± s.e.
n = number of animals.
* = significant at $P<0.05$, ** = significant at $P<0.01$, *** = significant at $P<0.001$ as compared to the control values.

7-AOA was inactive. Glycine produced a marginal but statistically significant fall in blood pressure only on the first day of treatment. The antihypertensive effects of GABA, DAVA and EACA, in contrast to those of α-methyldopa and guanethidine, were of relatively short duration. A significant decrease in blood pressure was seen 2 hr after the first administration of GABA and DAVA while 22 hr later blood pressure had returned to hypertensive levels. A similar fall in blood pressure was measured 2 hr after administration on the third and fourth day of treatment. The antihypertensive effect of EACA seemed to develop more slowly. Decreases in blood pressure were seen only 2 hr after administration on the third and fourth day of treatment (Table 1).

Noradrenaline concentrations in heart and brain of renal hypertensive rats. The noradrenaline concentrations in heart and brain of animals

treated intraperitoneally with the ω-amino-acids or with guanethidine (subcutaneously) or α-methyldopa (orally) were determined. The results are shown in Table 2. Glycine, GABA and 7-AOA did not influence myocardial catecholamine concentrations. EACA, on the other hand, produced a marked depletion which surpassed that produced by α-methyldopa or guanethidine. DAVA reduced myocardial catecholamine content slightly. A decrease in brain catecholamine concentration was produced only by α-methyldopa.

TABLE 2. NORADRENALINE CONCENTRATIONS IN HEART AND BRAIN OF RENAL HYPERTENSIVE RATS

4 days treatment with	Noradrenaline μg/g wet weight			
	n	Heart	n	Brain
0·9% NaCl	17	0·76±0·031	17	0·410±0·015
Guanethidine 3 mg/kg/day s.c.	6	0·14±0·015***	6	0·366±0·031
α-Methyldopa 300 mg/kg/day oral	4	0·16±0·010***	4	0·114±0·006***
Glycine 1000 mg/kg/day i.p.	2	0·79	3	0·407±0·018
GABA 1000 mg/kg/day i.p.	3	0·68±0·120	3	0·431±0·023
DAVA 1000 mg/kg/day i.p.	6	0·48±0·036**	6	0·382±0·020
EACA 1000 mg/kg/day i.p.	3	0·099±0·030***	3	0·431±0·056
7-AOA 1000 mg/kg/day i.p.	3	0·55±0·110	3	0·398±0·017

Values are means ± s.e.
n = number of extracts.
** = $P<0.01$, *** = $P<0.001$ as compared to the control values.

Influence of a single dose of EACA on blood pressure in renal hypertensive rats and myocardial catecholamine stores in normotensive rats. As seen in the previous experiment, a single intraperitoneal dose of EACA had no influence on blood pressure (Table 3). A progressive decrease in blood pressure seen after a single injection of EACA was also found in saline-treated control animals, and is probably the result of repeated ether anaesthesia. Myocardial catecholamine levels, however, decreased

TABLE 3. EFFECT OF A SINGLE INTRAPERITONEAL INJECTION OF EACA ON BLOOD PRESSURE IN RENAL HYPERTENSIVE RATS

Treatment	n	Initial blood pressure mm Hg	Decrease in blood pressure (Δ mm Hg) after				
			2 hr	4 hr	6 hr	8 hr	14 hr
0·9% NaCl 2 ml/kg i.p.	13	233±4	−20±5	−34±4	−41±6	−40±5	−21±5
EACA 1000 mg/kg i.p.	13	210±3	−27±6	−39±6	−36±9	−42±8	−18±10

Values are means ± s.e.
n = number of animals.

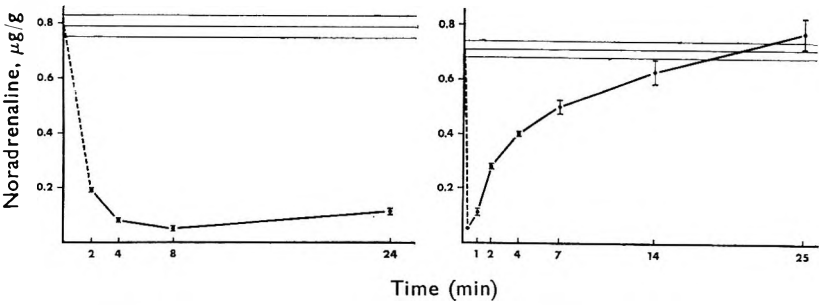

FIG. 1. Effect of a single intraperitoneal injection of 1000 mg/kg EACA on myocardial noradrenaline concentrations in normotensive rats. Points represent means of 3–5 extracts of two hearts each; vertical bars s.e. Control values are given as horizontal lines.

rapidly: the lowest concentrations were found 4–24 hr after administration. Within 4 days the catecholamine levels had been restored to about 50% of control values, and full recovery was found after 3 weeks (Fig. 1).

Pressor responses to noradrenaline and physostigmine. Potentiation of the noradrenaline effect and inhibition of the pressor response to physostigmine was produced by guanethidine, α-methyldopa and EACA. Glycine, GABA, DAVA and 7-AOA had no influence on the pressor responses (Table 4).

TABLE 4. PRESSOR RESPONSES TO NORADRENALINE AND PHYSOSTIGMINE IN NORMOTENSIVE RATS

4 days treatment with	n	Pressor response (Δ mm Hg) to	
		Noradrenaline 6 μg/kg i.v.	Physostigmine salicylate 150 μg/kg i.v.
0·9% NaCl	12	58±4	37±3
Guanethidine 3 mg/kg/day s.c.	9	70±4*	22±5*
0·9% NaCl	18	58±3	45±4
α-Methyldopa 300 mg/kg/day oral	20	72±4***	32±3*
0·9% NaCl	10	40±4	52±5
Glycine 1000 mg/kg/day i.p.	5	34±8	61±12
GABA 1000 mg/kg/day i.p.	10	34±3	42±6
0·9% NaCl	7	45±3	32±7
DAVA 1000 mg/kg/day i.p.	10	54±4	25±4
0·9% NaCl	10	40±4	52±5
EACA 1000 mg/kg/day i.p.	9	57±5*	35±2***
7-AOA 1000 mg/kg/day i.p.	5	36±4	51±5

Values are means ± s.e.
n = number of animals.
* = significant at $P<0.05$, ** = significant at $P<0.01$, *** = significant at $P<0.001$.

Discussion

Obianwu (1967a, b) showed that EACA, like guanethidine, inhibits the dual amine uptake-concentration mechanisms of the adrenergic neurons. He also pointed out that the adrenergic nerve blockade induced by EACA differs from that produced by guanethidine. The short-lasting antihypertensive effect of EACA would tend to support this suggestion. EACA is absorbed and eliminated rapidly in man (Dupont, 1965). Assuming that this is also true in the rat, variations in the intensity of the antihypertensive effect may reflect variations in blood EACA concentrations.

Myocardial catecholamine levels, however, do not vary to such degree. A single intraperitoneal injection of EACA reduced myocardial catecholamine stores in normotensive rats for at least 24 hr to approximately the same extent as 4 days treatment of hypertensive rats. The catecholamine depletion found 4 and 8 hr after 1000 mg/kg intraperitoneally was slightly more pronounced than that which Lippmann & others (1965) have described after oral administration of 500 mg/kg. It is more marked than that produced by guanethidine or α-methyldopa. In contrast, blood pressure was not significantly influenced by a single intraperitoneal injection of EACA. Subsequent to an initial sympathomimetic phase, EACA produced a 20% decrease in blood pressure 6 hr after administration of 2 g/kg i.p. in the normotensive, non-anaesthetized rat (Andén, Henning & Obianwu, 1968). In our experiments neither an initial increase in blood pressure nor a later decrease in blood pressure was observed. These differences may be due to the differing EACA doses used or to the influence of the light ether anaesthesia, which decreased blood pressure in saline-treated control rats.

DAVA reduced the concentration of myocardial catecholamines by about 30% after 4 days treatment. None of the other ω-amino-acids studied influenced myocardial catecholamine contents significantly. Likewise, it has been shown that β-alanine does not reduce myocardial catecholamine concentrations (Lippmann & others, 1965).

Inhibition of adrenergic transmission as estimated by reduction of the pressor response to physostigmine, was seen only after EACA, α-methyldopa and guanethidine. According to the results of Varagić (1955), the pressor response to physostigmine in the urethane-anaesthetized rat may be attributed to central sympathetic stimulation. A reduction in this pressor response has been related to inhibition of central sympathetic centres or impairment of peripheral sympathetic transmission (Lešić & Varagić, 1961). The potentiation of the pressor response to noradrenaline by EACA indicates that inhibition of adrenergic transmission is not the result of adrenergic α-receptor blockade.

GABA and DAVA, which had an effect on blood pressure similar to that of EACA, had little or no influence on myocardial catecholamine stores and did not affect the pressor response to physostigmine. Thus, GABA and DAVA seem to have a different mechanism of antihypertensive action. The other ω-amino-acids, glycine and 7-AOA, did not influence any of these parameters. The slight effect of glycine on blood

pressure on the first day of treatment is probably without pharmacological significance.

A single administration of EACA produced rapid and long-lasting depletion of myocardial noradrenaline stores. Likewise, Obianwu (1967a) reported an impairment of membrane and storage particle uptake mechanisms within a few hours after giving a single intraperitoneal dose of 1000 mg/kg EACA. The antihypertensive effect of EACA on the other hand, developed only after repeated injections and does not seem to be directly correlated with the depletion of peripheral catecholamine stores or with impairment of adrenergic neuron uptake-storage mechanisms.

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J. Pharm. Pharmac., 1968, **20**, 744–745 Received February 12, 1968

Inhibitory activity of 8β-carbobenzyloxyaminomethyl-1,6-dimethyl-10α-ergoline towards stimulant effects by 5-hydroxytryptamine and amphetamine on liver fluke, *Fasciola hepatica*, *in vitro*

C. BERETTA* AND A. LOCATELLI**

The powerful and long lasting anti-5-hydroxytryptamine effects of 8β-carbobenzyloxyaminomethyl-1-6-dimethyl-10α-ergoline (MCE) have been tested on the spontaneous rhythmical activity of *Fasciola hepatica*, *in vitro*. The stimulant effects of both 5-HT and amphetamine on this preparation were totally blocked for at least 2 hr by 0·01 μg/ml of MCE. One mole of MCE counteracts the effects of about 2×10^4 mole of 5-HT or amphetamine. A few other drugs exert inhibitory activity on this preparation but at far higher doses. A comparison between the data obtained for MCE and for bromolysergic acid diethylamide (BOL) reveals that MCE is a more potent inhibitory drug than BOL and that, moreover, it is characterized by total lack of depressant or paralysant effects.

THAT 5-hydroxytryptamine is a mediator of nerve action in certain invertebrates was suggested by Welsh (1953). Mansour (1957) showed that 5-HT stimulated muscular contractions both in intact and in degangliated *Fasciola hepatica*, *in vitro*, and that bromolysergic acid diethylamide (BOL) antagonized the stimulant effects not only of 5-HT but also of lysergic acid diethylamide and amphetamine. Compounds such as BOL, harmine and yohimbine were therefore considered likely to depress motility in the intact liver fluke by combining with tryptamine receptors, thus blocking by competition the site of action of endogenously released transmitter.

The similarity between the effects of amphetamine and 5-HT and the blocking action exerted by BOL on these effects were considered to be evidence that these amines may act on the same receptors (Mansour, 1957). These assumptions led us to examine the effects on the liver fluke of 8β-carbobenzyloxyaminomethyl-1-,6-dimethyl-10α-ergoline (MCE), whose strong and long lasting antagonism to 5-HT was reported both *in vitro* (Beretta, Ferrini & Glässer, 1965) and *in vivo* (Beretta, Glässer & others, 1965). From the literature it was not clear whether the tonic contractions seen after the administration of 5-HT or amphetamine were caused directly or whether these were only indirectly derived through a primary release of other transmitter agents such as catecholamines. We report some experiments to investigate this problem.

Experimental

MATERIALS AND METHODS

Flukes were obtained from bile ducts of bovine livers within 1 hr of the death of the host. They were washed and subsequently placed in Ringer solution (NaCl 0·9; $CaCl_2$ 0·006; KCl 0·04; $NaHCO_3$ 0·05; glucose 0·05 g in 100 ml of distilled water at 37°). The flukes were

From * the Institute of Pharmacology, and ** the Institute of Parasitology, Faculty of Veterinary Medicine, University of Milan, Italy.

attached to an isotonic lever under slight tension according to Chance & Mansour (1949), and tracings were recorded on a kymograph. Degangliated preparations were obtained by cutting off the head of the worm just below the ventral sucker. The stimulant drugs were added to the bath and left in contact with the preparations for 2–3 min and then removed by washing with fresh Ringer solution.

After recording the spasmogenic effect of a drug, a period of at least 20–25 min was allowed to elapse before the administration of another drug. Inhibitory drugs were administered similarly, but the time contact with the preparation was always 15 min, after which the drug was removed and a new stimulant compound was administered.

DRUGS

Amphetamine sulphate, 5-hydroxytryptamine creatinine sulphate, dihydroergotamine tartrate, phentolamine methansulphonate (CIBA), adrenaline hydrogen tartrate, propranolol hydrochloride (ICI) and 2-bromolysergic acid diethylamide were dissolved in distilled water. MCE was dissolved in warm distilled water with the aid of a slight excess of maleic acid.

Results

Both 5-HT and amphetamine stimulated the intact parasites and degangliated preparations. The threshold doses of 5-HT (as creatinine sulphate) and amphetamine sulphate were 1–10 μg/ml ($0{\cdot}25$–$2{\cdot}46 \times 10^{-5}$ M) and 5–10 μg/ml ($1{\cdot}35$–$2{\cdot}70 \times 10^{-5}$ M) respectively.

No latency was observed between the administration of the drug and the start of the tonic contraction. After washing, when the drug was removed, tone subsided but contractions of higher amplitude and of faster frequency than normal appeared. At this time the administration of another stimulant drug elicited only a slight effect or no effect at all. This was observed both with 5-HT administered after 5-HT or amphetamine and with amphetamine administered after amphetamine or 5-HT. A period of 20–25 min between two successive administrations of stimulants was necessary to restore the original sensitivity. Nevertheless tachyphylaxis to 5-HT was often persistent and when this occured the experiment was stopped. In experiments evaluating antagonists, 5-HT and amphetamine (agonists) were always administered at doses of 200 μg/ml; these were active in 75–80% of the experiments.

In Table 1 are summarized the molar concentrations of MCE, dihydroergotamine, phentolamine and propranolol which inhibited the stimulant effects of 5-HT and amphetamine. Also included are the results of some experiments we made using BOL and those reported by Mansour (1957) for the same compound. The inhibitory effects of dihydroergotamine, phentolamine and propranolol were short lasting (20–40 min) in spite of the high dosage, and a good recovery of the effects of the two agonists was obtained after the first or second addition of them to the bath after the antagonist.

TABLE 1. ANTAGONISM EXERTED BY BOL, MCE, DIHYDROERGOTAMINE (DHE), PHENTOLAMINE AND PROPRANOLOL AGAINST THE SPASMOGENIC EFFECTS OF 5-HYDROXYTRYPTAMINE AND AMPHETAMINE ON *Fasciola hepatica in vitro*

Stimulant drugs (molar conc.)	Antagonists (molar conc.)				
	BOL	MCE	DHE	Phentol-amine	Propranolol
5-Hydroxytryptamine creatinine-sulphate 5×10^{-4}	$0{\cdot}2 \times 10^{-6}$ $0{\cdot}5 \times 10^{-6}$ $1{\cdot}0 \times 10^{-6}$ (*)	$2{\cdot}4 \times 10^{-8}$	$3{\cdot}7 \times 10^{-5}$ $7{\cdot}5 \times 10^{-5}$	$2{\cdot}8 \times 10^{-4}$ $5{\cdot}7 \times 10^{-4}$	$6{\cdot}7 \times 10^{-4}$
Amphetamine sulphate 5×10^{-4}	$0{\cdot}2 \times 10^{-6}$ $0{\cdot}5 \times 10^{-6}$ $1{\cdot}0 \times 10^{-6}$ (*)	$2{\cdot}4 \times 10^{-8}$	$3{\cdot}7 \times 10^{-5}$ $7{\cdot}5 \times 10^{-5}$	$2{\cdot}8 \times 10^{-4}$ $5{\cdot}7 \times 10^{-4}$	$6{\cdot}7 \times 10^{-4}$

(*) Data reported by Mansour (1957).

We found the inhibitory effects of BOL to occur at doses a little lower than those described by Mansour (1957); removal of the drug from the organ bath promptly restored a good sensitivity of the preparations towards the two amines. BOL always exerted a depressant action on the spontaneous rhythmical motality of the worms just as was previously described by Mansour (1957). In contrast, MCE in low concentration counteracted the effects of 5-HT or amphetamine for a long period

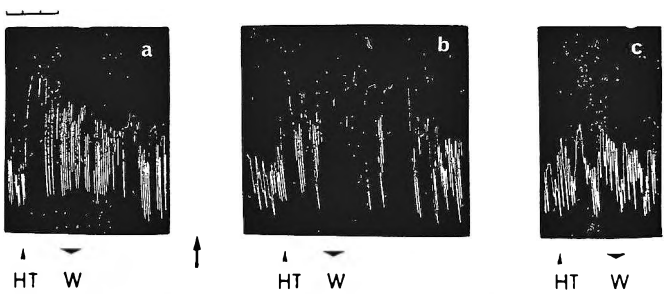

FIG. 1. Effects of 5-hydroxytryptamine creatinine sulphate (HT) 200 μg/ml administered before (a), 20 min (b) and 94 min (c) after 0·01 μg/ml of MCE at the arrow. The time contact of MCE was 15 min. At W washout. Time scale = 3 min.

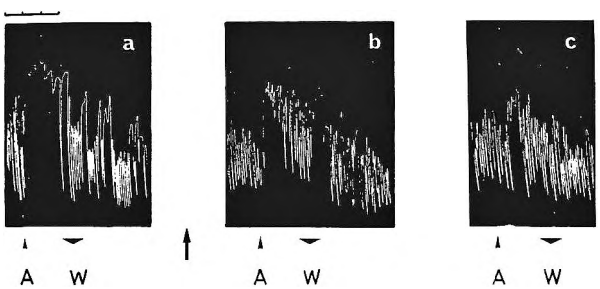

FIG. 2. Effects of amphetamine sulphate (A) 200 μg/ml administered before (a), 20 min (b) and 94 min (c) after 0·01 μg/ml of MCE at the arrow. The time contact of MCE was 15 min. At W washout. Time scale = 3 min.

(Figs 1 and 2). In some experiments 0·01 μg/ml of MCE caused an inhibition that could not be overcome for more than 2 hr. The first dose of 5-HT after antagonist did not elicit an inhibitory effect but on the contrary showed potentiation.

TABLE 2. THE RATIOS BETWEEN THE MOLAR CONCENTRATION OF THE ANTAGONISTS AND THE AGONISTS 5-HT AND AMPHETAMINE

BOL	MCE	DHE	Phentolamine	Propranolol
1:1000	1:20161	1:13·2	1:1·74	1:0·83
1:2500		1: 6·6	1:0·87	
1:500 (*)				

(*) Ratio calculated on the base of the results of Mansour (1957).

MCE alone, at doses up to 1 μg/ml, did not affect the spontaneous motility of the liver fluke. No difference was found between the doses of antagonist needed to inhibit the effects of 5-HT or amphetamine. In Table 2 the ratios between the blocking molar concentrations of the inhibitory drugs and the molar concentrations of the stimulant drugs are summarized. The data in the first column have been calculated from both our results and those of Mansour (1957) with BOL, and it can be seen that one mole of MCE counteracts about 2×10^4 mole of 5-HT or amphetamine.

Discussion

The similarity of the effects elicited by 5-HT and amphetamine, and the evidence that these effects could be blocked by inhibitory drugs at the same dose level, suggests that the two amines probably act at the same receptor sites, which are peripheral and independent from the peri-ventosal ganglia. MCE proved to be the most powerful of the inhibitory drugs used, the ratio of its molar concentration to that of the agonists 5-HT and amphetamine being $1:2 \times 10^4$. The ratios for BOL were for 5-HT $1:1 \times 10^3$ and for amphetamine $1:2{\cdot}5 \times 10^3$ [the ratio calculated from the results of Mansour (1957) is $1:5 \times 10^2$]; for dihydroergotamine the respective figures were 1:13·2, 1:6·6; phentolamine 1:1·7, 1:0·9; propranolol 1:0·8.

These results strongly support the opinion that two amines act at a single tryptamine receptor (Mansour, 1957). It is noteworthy that at a dose of 0·01 μg/ml MCE strongly blocked the effects of 5-HT, whereas at a dose hundred times greater it is without effect in blocking the spontaneous movements of the fluke.

In this respect, MCE behaves quite differently from BOL which depresses spontaneous movements at doses 10 times smaller than those effective against 5-HT. The difference between BOL and MCE suggests that BOL is active at this site, or alternatively exerts a non-specific depression on the fluke, whereas MCE would appear to be active only against exogenous 5-HT and to be ineffective against endogenous transmitter. The last suggestion involves the conclusions of Welsh (1953) about 5-HT as a mediator of neuromuscular transmission in invertebrates.

The potentiation of the effects of the first dose of 5-HT after MCE, resembling the results obtained with this anti-5-HT compound on isolated rat uterus *in vitro* (Beretta, Ferrini & Glässer, 1965), may suggest action at true receptors and indicates that uptake on the receptors is slow since later on there is a long-lasting blockade.

Acknowledgements. We are grateful to the Veterinary Medical Doctors of the slaughter-house of Milan for their help in collecting the liver flukes and to Prof. V. Erspamer for a supply of BOL and to Prof. A. H. Glässer of the Farmitalia Research Laboratories for a supply of MCE. Moreover we thank Vister (Vismara Terapeutici) for a generous supply of propranolol hydrochloride (ICI).

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J. Pharm. Pharmac., 1968, **20**, 749–753 Received February 14, 1968

Distribution of prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$, in some animal tissues

S. M. M. KARIM, K. HILLIER AND JEAN DEVLIN*

A survey of the distribution of prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ in fourteen tissues from the dog, cat, rat, rabbit, guinea-pig and chicken has been made. One or more of these prostaglandins are present in varying amounts in most tissues with PGE_2 $PGF_{2\alpha}$ occurring most commonly.

SIX of the naturally occurring "primary" prostaglandins (E_1, E_2, E_3, $F_1\alpha$, $F_2\alpha$, $F_3\alpha$) have been described and the elucidation of their structure has been fully documented and recently reviewed by Bergström & Samuelsson (1965) and Samuelsson (1965). Five of these primary prostaglandins have been isolated from human seminal fluid (Bergström & Samuelsson, 1962; Samuelsson, 1963). Prostaglandins do not only occur in male accessory glands and their secretions. They have been found in thymus, pancreas, brain and kidney (Bergström & Samuelsson, 1965; Samuelsson, 1965). They have also been isolated from human menstrual fluid (Eglinton, Raphael & others, 1963) and from human umbilical cord and amniotic fluid (Karim, 1966, 1967; Karim & Devlin, 1967). Karim,

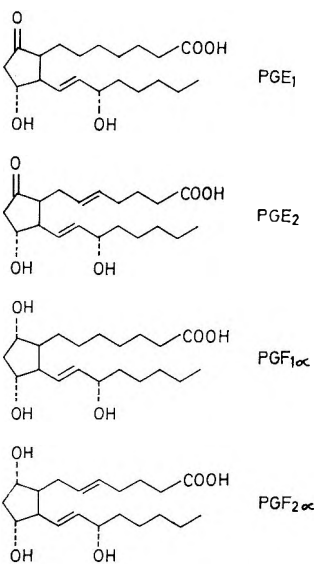

FIG. 1. Formulae of four naturally occurring prostaglandins.

From The Department of Pharmacology and Therapeutics, Makerere University Medical School, P.O. Box No. 7072, Kampala, Uganda.

* Present Address: Department of Pharmacology, Harvard Medical School, 25, Shattuck St, Boston, Mass., U.S.A.

Part of the work presented in this paper was carried out at The Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, University of London, Goldhawk Road, London, W.6.

Sandler & Williams (1967) recently observed prostaglandins to be present in 16 of 23 different human tissues examined. The present investigation is concerned with the distribution of prostaglandins E_1, E_2, $F_1\alpha$ and $F_2\alpha$ in tissues from six widely used laboratory animals.

Experimental

MATERIAL AND METHODS

The animals used were 12 Wistar rats of either sex, 120–150 g; 6 male guinea-pigs, 500–800 g; 3 adult chickens; 3 male rabbits; 2 male cats; 1 male greyhound. The rats, guinea-pigs and chickens were stunned and bled out, and the dog, cats and rabbits were intravenously infused with sodium pentobarbitone until respiration ceased. The selected tissues were removed immediately, pooled for each species and stored at $-10°$ until extracted. The prostaglandins were extracted by the method of Samuelsson (1963). The methods used for the chromatographic separation and identification of the prostaglandins were as described by Karim (1967). Briefly, this consisted of group separation of the E prostaglandins from the F series by chromatography on a column of silicic acid followed by separation of the individual prostaglandins by thin-layer chromatography in solvent system A11 of Green & Samuelsson (1963) using plates coated with silica gel G and containing 7·5% silver nitrate. Markers of known prostaglandins were used. The activity on the plate was located by biological assay. Biological assay of the prostaglandins was on the isolated ascending colon preparation from the jird, *Meriones libycus*. The colon was removed and set up in a 4 ml organ bath of de Jalon solution at 30°, and gassed with oxygen (Karim & others, 1967). Isotonic contractions were recorded on smoked kymograph paper. A dose cycle of 4–5 min with a contact time of $1\frac{1}{2}$–2 min was used depending on the response of the jird preparation. For any one assay, the contact time was constant. The amounts of prostaglandins present in tissue extracts were estimated by the bracket assay method using pure synthetic prostaglandins as standards. For the estimation of some of the extracts the guinea-pig isolated proximal colon preparation (Karim, 1967) was also used.

Results

The survey of different tissues from the six species revealed the presence of the four prostaglandins in varying amounts in most tissues. Identification was based on several characteristics of these substances, including the behaviour of the active material as polar lipid acids on partition between ethyl acetate and water, and also chromatographic properties. Full details of the methods of identification are given by Karim (1967).

The quantitative estimation of small amounts of prostaglandins present in some of the tissues examined has been made possible by the use of the jird isolated colon preparation. Table 1 shows the threshold concentrations of the four prostaglandins required to elicit the contraction of the preparation and the ratio of their biological activity on 60 preparations.

TABLE 1. RATIO OF BIOLOGICAL ACTIVITY OF PROSTAGLANDINS E_1, E_2, $F_1\alpha$ AND $F_2\alpha$ AND THEIR THRESHOLD DOSE ASSESSED USING 60 PREPARATIONS OF THE JIRD ISOLATED COLON. THE ACTIVITY OF E_1 IS TAKEN AS 1.

Prostaglandins	Ratio of activity	Range of threshold doses ng/ml
E_1	1	0·2 –0·7
E_2	2	0·05–0·4
$F_1\alpha$	0·2	1·0 –4·0
$F_2\alpha$	1·5	0·3 –0·5

The concentrations of prostaglandins in ng/g wet weight of tissue, uncorrected for recovery are shown in Table 2. The recovery of known amounts of pure prostaglandins added to various tissues, including those that did not contain any prostaglandins, which were subjected to the standard extraction and separation procedures, was found to be 59% ± 12% (5 experiments).

TABLE 2. DISTRIBUTION (NG/G OF TISSUE) OF PROSTAGLANDINS IN ANIMAL TISSUES

Tissue	Animal					
	Dog	Cat	Rat	Guinea-pig	Rabbit	Chicken
Heart	ND	3·7 $F_2\alpha$	3·8 E_2	ND	ND	3·5 $F_2\alpha$ 51·0 E_2
Thymus	2·5 E_1 2·0 E_2 4·0 $F_2\alpha$	4·0 E_1 5·1 E_2 3·3 $F_2\alpha$	17·5 E_1	6·0 $F_2\alpha$	1·0 $F_2\alpha$	25·8 E_2
Skeletal muscle	1·0 E_2 2·7 $F_2\alpha$	1·0 E_1	4·4 E_2 2·1 $F_2\alpha$	1·1 E_2 1·1 $F_2\alpha$	ND	1·8 $F_2\alpha$
Submaxillary salivary glands	1·9 E_2 1·9 $F_2\alpha$	0·75 E_2 1·1 $F_2\alpha$	9·2 E_2 6·9 $F_2\alpha$	4·7 E_2 7·8 $F_2\alpha$	17·5 E_2 2·7 $F_2\alpha$	NE
Spleen	0·4 E_1 18·7 E_2 2·3 $F_2\alpha$	8·75 E_2	37·5 E_2 8·0 $F_2\alpha$	4·0 E_2 6·1 $F_2\alpha$	23·2 E_2 20·0 $F_2\alpha$	180·2 E_2 60·6 $F_2\alpha$
Adrenals	5·0 E_2	80·0 E_2 101·7 $F_2\alpha$	ND	ND	42·0 E_2	40·1 E_2 250·9 $F_2\alpha$
Sympathetic chain	31·7 E_2 57·5 $F_2\alpha$	37·5 E_2 25·6 $F_2\alpha$	NE	NE	NE	NE
Pancreas	1·45 E_2 1·1 $F_2\alpha$	3·3 E_2 3·2 $F_2\alpha$	18·2 E_2 9·7 $F_2\alpha$	ND	5·5 E_2 2·4 $F_2\alpha$	12·5 E_2 10·0 $F_2\alpha$
Thyroid	ND	20·0 E_2 6·0 $F_2\alpha$	154·4 E_2 162·1 $F_2\alpha$	480·8 E_2 160·9 $F_2\alpha$	133·0 E_2 1·83 $F_2\alpha$	192·3 $F_2\alpha$ 160·4 E_2
Kidney	ND	ND	50·4 E_2 9·0 $F_2\alpha$	28·5 E_2 20·2 $F_2\alpha$	32·0 E_2 18·2 $F_2\alpha$	9·2 $F_2\alpha$
Liver	ND	ND	18·2 E_2 9·4 $F_2\alpha$	12·0 $F_2\alpha$	1·75 E_2 8·50 $F_2\alpha$	ND
Blood	ND	ND	ND	ND	ND	ND
Vagus	45·3 E_2 30·4 $F_2\alpha$	90·9 E_2 45·0 $F_2\alpha$	NE	NE	NE	NE
Lungs	ND	65 E_2, 15·5 $F_2\alpha$	16·6 E_2 90·4 $F_2\alpha$	2·5 E_2 375·0 $F_2\alpha$	5·4 E_2, 8 $F_2\alpha$	7·7 E_2 30·4 $F_2\alpha$

ND—Prostaglandins E_1, E_2, $F_1\alpha$, $F_2\alpha$ not detected.
NE—Tissue not extracted.

In addition to the prostaglandins identified in the different tissues, some extracts of rabbit pancreas, cat liver and guinea-pig lung contained other

polar lipid-soluble acidic smooth muscle stimulating substances, which had Rf values, obtained by biological assay, of 0·2–0·3, which were lower than that of $PGF_2\alpha$ (0·45–0·50) on the thin-layer plates. It is conceivable that this activity was due to the presence of PGE_3 or $PGF_3\alpha$. However, pure samples of these two prostaglandins were not available and hence no further attempt was made to identify the substances.

Discussion

Published studies on the distribution of prostaglandins show that there are species differences. Prostaglandins are present in very high concentration in semen of man and sheep but the semen of a number of other species contains none (Bergström, 1963; Bergström & Samuelsson, 1962; Samuelsson, 1966). The presence of prostaglandins has been reported in the dog spleen (Davies, Horton & Withrington, 1966) and the kidney (Lee, Covino & others, 1965). However, human spleen and kidney do not contain any prostaglandins (Karim & others, 1967).

The results of the present investigation show that although the four prostaglandins are widely distributed amongst animal tissues there are differences in their concentrations between similar tissues in different species. Most chicken tissues examined, apart from the liver, contain larger amounts of the prostaglandins than the same tissues from other species. Horton & Main (1967) have reported that concentrations of prostaglandins in the central nervous tissues of the chicken are higher than those in the similar tissues from the dog or the cat. Liver, kidney, heart and thyroid from the dog, the liver and kidney from the cat, rat adrenals, guinea-pig heart and pancreas, rabbit heart and skeletal muscle and chicken liver contain no detectable amounts of the four prostaglandins. In no species were they detected in the blood.

Paasonen (1958), studying the 5-hydroxytryptamine (5-HT) content of rat, dog and rabbit thyroids, reported the presence of a smooth muscle stimulating substance in the rat thyroid which like prostaglandins was soluble in 90% acetone. This activity was shown not to be due to histamine, 5-HT or acetylcholine. Garven (1956) reported a substance with similar properties in extracts or rabbit thyroid. Since prostaglandins E_2 and $F_2\alpha$ have been identified in the thyroids of both these species in moderately high concentrations (Table 2) it is conceivable that the reported activity could well have been due to them. No such smooth muscle stimulating activity was observed in the dog thyroid and we did not detect prostaglandins in dog thyroid.

Prostaglandins were found in the submaxillary glands of all species studied. Stimulation of chorda tympani nerve leads to vasodilatation in these glands in the dog, cat and rabbit (Morley, Schachter & Smaje, 1963; Bhoola, Morley & others, 1965; Schachter, 1966). The cause of this vasodilatation is in dispute. Kallikrein has been suggested to be the vasodilator substance by Hilton & Lewis (1958), Lewis (1959) and Hilton (1960, 1966), but other workers believe that it plays no significant role in this action (Bhoola & others, 1965; Schachter, 1966). Schachter

(1966) suggests that "neurotransmitter substance released in close relation to the receptor sites in the vascular muscle is concerned". The finding that prostaglandins are intense vasodilators in most vascular beds (Horton & Main, 1963; Ånggärd & Bergstrom, 1963; Nakano & McCurdy, 1967) and that they can be released upon nerve stimulation (Ramwell & Shaw, 1963; Ramwell, Shaw & Kucharske, 1965) and that they occur in the submaxillary glands of the six species investigated would make further work in their role as vasodilators in the salivary glands of interest.

Acknowledgement. We are grateful to Dr. J. E. Pike (Upjohn Co., Kalamazoo, Michigan) for a gift of pure prostaglandins.

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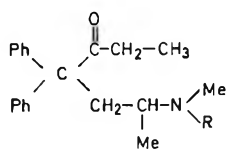
J. Pharm. Pharmac., 1968, **20**, 754–762 Received February 27, 1968

The biotransformation of methadone in man: synthesis and identification of a major metabolite

A. H. BECKETT, J. F. TAYLOR, A. F. CASY* AND M. M. A. HASSAN*

After administration of methadone to man, only two basic urinary excretion products were detected, these were unchanged drug and 2-ethyl-1,5-dimethyl-3,3-diphenyl-1-pyrroline. The hydriodide of the latter was synthesized and its endocyclic alkene structure was confirmed from infrared and nuclear magnetic resonance spectral data. This compound was shown to be identical with the hydriodide of the basic product formed by reaction of 1,5-dimethyl-3,3-diphenylpyrrolid-2-one with ethyl-lithium.

METHADONE (Ia) may be metabolized to a *N*-demethyl derivative (Ib). An unidentified basic metabolite of methadone was found in the bile of rats receiving the drug (Way, Signorotti & others, 1951). This metabolite partitioned from organic solvents into acetate buffer (pH 3·6)



Ia R = Me
b R = H

more readily than methadone and contained most of the methadone molecule (Miller & Elliott, 1955). Methadone is *N*-demethylated *in vitro*, since incubation with rat and rabbit liver microsomal preparations liberated formaldehyde (Axelrod, 1956). Vidic (1957) reported evidence for *in vivo* *N*-demethylation from the presence of a primary and secondary amine in paper chromatograms of urinary excretion products of methadone. Attempts to synthesize *N*-demethylmethadone failed (Harper, Jones & Simmonds, 1966); however, Pohland, on treating 1,5-dimethyl-3,3-diphenylpyrrolid-2-one (VI) with ethyl-lithium obtained a basic product with an infrared spectrum similar to that of the metabolite isolated by Way (private communication quoted by Way & Adler, 1962). The basic product was assumed, but not established, to be the tertiary alcohol VII or the alkene VIII as illustrated (flow sheet 2). It was postulated that the pyrrolidine structure arises as a result of cyclization of the secondary amine produced by *N*-demethylation of methadone.

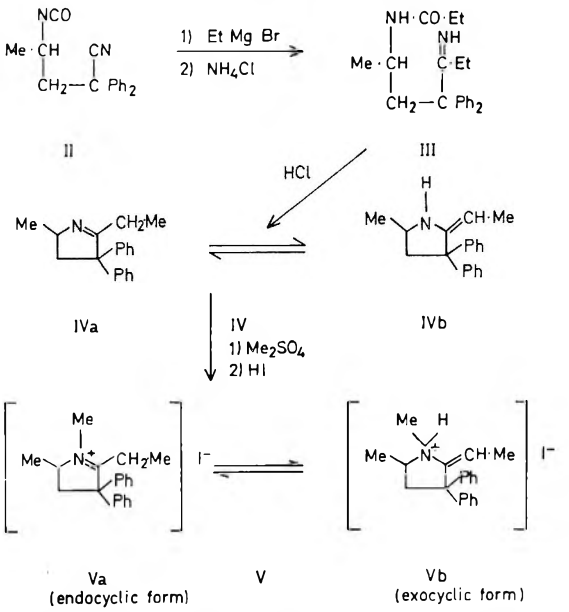
The structure of the product, obtained from the reaction of the pyrrolidone VI with ethyl-lithium, is now established unequivocally; and its physico-chemical characteristics are compared with those of a major metabolite of methadone excreted in man.

From the Department of Pharmacy, Chelsea College of Science and Technology (University of London), London S.W.3, England.
* Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada.

Experimental

METHOD 1 (Flow Sheet 1)

Reaction of 3-cyano-1-methyl-3,3-diphenylpropylisocyanate and ethylmagnesium bromide and product hydrolysis. 3-Cyano-1-methyl-3,3-diphenylpropyl isocyanate (II—Bretschneider, Klötzer & others, 1958) (19·32 g) in dry toluene (250 ml) was added to ethylmagnesium bromide in ether (150 ml), prepared from ethyl bromide (61 g) and magnesium (13·4 g).



Flow sheet 1. (see Bretschneider & others, 1959).

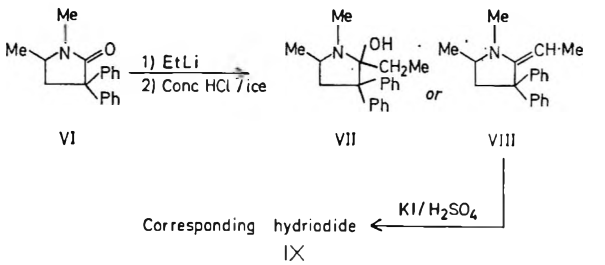
The ether was distilled off, the mixture heated under reflux for 3 hr and the reaction mixture added to a cold saturated solution of ammonium chloride. The organic layer was separated and the aqueous layer extracted with ether. The ethereal extracts, together with the organic layer, were dried over anhydrous sodium sulphate, filtered and evaporated to give the ketimine (III) as the crude base (20 g). The crude base (III) (20 g) and concentrated hydrochloric acid (60 ml) were heated under reflux for 2 hr. The base recovered from the acidic solution was distilled under high vacuum to give the base IV (9·5 g), b.p. 135—140°/0·3 mm (Bretschneider & others, 1958, b.p. 135–140°/0·3 mm) (Found: C 86·3; H, 8·05; N, 5·4. $C_{19}H_{21}N$ requires C, 86·6; H, 8·0; N, 5·3%). It formed a hydrochloride, m.p. 160–162°, from ethanol-ether (Found: C, 75·1; H, 7·4; N, 4·9. $C_{19}H_{22}ClN$ requires: C, 76·1; H, 7·4; N, 4·7%).

Reaction of the base IV with dimethyl sulphate. The base IV (5·2 g) in benzene (50 ml) and dimethyl sulphate (2·6 g) were heated under reflux for 4 hr. The solvent was distilled off under reduced pressure on a water bath, water (40 ml) added and the resultant turbid solution clarified with activated charcoal. Potassium iodide (20 g) was added to the clear solution and

the precipitated solid recrystallized from aqueous ethanol to give the hydriodide V (6 g), m.p. 150–152°. It had the following nmr characteristics (cycles/sec from tetramethylsilane in $CDCl_3$): 443 main peak of multiplet (10 aromatic protons); 307 quartet (C*H* Me); 240, singlet (N Me); 95, doublet J7 (s-Me); 43 triplet J7·5 (CH_2*Me*). The base obtained from the iodide V was a yellow oil b.p. 130°/0·1 mm (Bretschneider & others, 1959, report m.p. 150–154° for the hydriodide and b.p. 130°/0·1 mm for the base), which became red in contact with air and heat.

METHOD 2 (Flow sheet 2)

Reaction of 1,5-*dimethyl*-3,3-*diphenylpyrrolid*-2-*one with ethyl-lithium.* Lithium (0·7 g), hammered out to a thin sheet and cut into strips, was placed in a flask containing ether (60 ml), cooled to −40° (Cardice-bath) and a slow stream of nitrogen passed through the apparatus. Ethyl bromide (5·5 g) was added dropwise, with stirring, at such a rate as to maintain the temperature at −40°. After 4 hr, when all the ethyl bromide had been



Flow sheet 2.

added, 1,5-dimethyl-3,3-diphenyl pyrrolid-2-one (VI-Gardner, Easton & Stephens, 1948; Walton, Ofner & Thorp, 1949) (6·6 g) in benzene (45 ml) and ether (75 ml) was added dropwise while allowing the reaction mixture to attain room temperature and the mixture heated under reflux for 30 min. The reaction mixture was cooled and added to ice and concentrated hydrochloric acid (20 ml). The base, recovered from the aqueous phase, was distilled under high vacuum to give the proposed 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine (VIII) (5 g), b.p. 130–131°/0·1 mm (Found: C, 85·2; H, 8·3; N, 4·8. $C_{20}H_{24}N$ requires: C, 86·6; H, 8·4; N, 5·05%). Potassium iodide (6 g) in water (6 ml) was added to the base VIII (2 g) dissolved in dilute sulphuric acid (20 ml) and the solid obtained recrystallized from aqueous ethanol to give the corresponding hydriodide (IX) m.p. and mixed m.p. 151–153° (Found: C, 59·05; H, 6·3; N, 3·4. $C_{20}H_{24}IN$ requires C, 59·3; H, 6·0; N, 3·5%).

PHYSICAL MEASUREMENTS

In chemical and metabolic studies, infrared spectra were recorded with a Unicam SP200 infrared spectrometer (solids as Nujol mulls and liquids as films). Nmr spectra of compound V in $CDCl_3$ were recorded with Perkin Elmer R10 and Varian HA-100 high resolution nmr spectrometers.

The chemical shifts of the protons were assigned by deuteration and spin-spin decoupling studies, using tetramethylsilane as internal standard.

Metabolic studies

The rate of excretion of weak acids and bases is dependent upon urinary pH (Milne, Scribner & Crawford, 1958; Weiner & Mudge, 1964 and references cited therein). Rendering the urine acidic increases the rate of excretion and recovery of basic compounds. This is considered to be due to the increased acidity of the glomerular filtrate which lowers the content of unionized compound and consequently the rate of reabsorption into the body by passive non-ionic diffusion. Therefore, we have given methadone to male volunteers whose urine was kept acidic. The excretion products were identified by comparing their gas-liquid and thin-layer chromatographic properties and their infrared absorption spectra with those of methadone and compound V.

Reagents. Redistilled analar diethyl ether and n-butanol. Hydrochloric acid, 5 N. Concentrated ammonia solution, sp. gr. 0·880.

GAS-LIQUID CHROMATOGRAPHY

A Perkin-Elmer F11 chromatograph equipped with a flame-ionization detector and a 0–5 mV Leeds and Northrup Speedomax G recorder, Model S were used. Chromatographic column A was glass tubing $\frac{1}{4}$ inch o.d., 2 m long, packed with 80–100 mesh Chromosorb G, acid-washed, treated with chlorodimethylsilane and coated with 2% w/w SE30. Chromatographic column B was stainless steel tubing, $\frac{1}{8}$ inch o.d., 1 m long, packed with 80–100 mesh Chromosorb G, washed and treated as above and coated with 5% w/w potassium hydroxide and 2% Carbowax 20M. The columns were kept for 24 hr under their operating conditions; oven temperature, 180° for Column A, and 185° for column B; injection-block temperatures, about 250°; hydrogen pressure, 14 lb/inch^2; air pressure, 25 lb/inch^2; nitrogen flow rate, 16 ml/min for Column A, 14 ml/min for Column B.

THIN-LAYER CHROMATOGRAPHY

Glass plates, 20 × 20 cm, were spread to a thickness of 0·5 mm with a

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF METHADONE, COMPOUND V AND THE URINARY EXCRETION PRODUCTS OF METHADONE

Solvent system	Rf Methadone	Rf Compound V	Spots shown up by Dragendorff's reagent and not appearing in control	
			Number	Rf
Ethanol (60) : acetic acid (30) : water (10) ..	0·46	0·31	2	0·46 0·30
Ethanol (50) : concentrated ammonia soln (5) : ethyl acetate (45)	0·73	0·75	1 (Long)	0·74
Methanol (60) : n-Butanol (15) : benzene (10) : water (15)	0·15	0·07	2	0·16 0·08
Ethanol (5) : dioxan (40) : benzene (50) : conc. ammonia soln (5)	0·75	0·79	2	0·79 0·75

mixture of Silica Gel G (Merck) and water (1 : 2). The plates were first allowed to dry at room temperature for 15 min and then heated for 2 hr at 80°. The solvent systems used are listed in Table 1.

PROCEDURE

(±)-Methadone (10 mg) was administered to four male volunteers whose urine was maintained acidic by oral ammonium chloride (Beckett & Tucker, 1966). Urine was collected 4 hr before (control) and 2–6 hr after drug administration (test). The control and test samples (100 ml) were adjusted to pH 9–11 with concentrated ammonia solution, extracted twice with n-butanol (½ vol) and the combined extracts evaporated to low bulk under reduced pressure.

Aliquots of test and control butanol concentrates, together with reference methadone and compound V, were applied separately to thin-layer plates. Chromatograms were developed at ambient room temperature in the solvent systems described in Table 1.

The butanol concentrates (5 ul) were also injected onto the two gas-liquid chromatographic columns. Similar injections were made with methadone and compound V as the free bases in butanol.

Urine excreted by one of the subjects was collected and pooled for the 48 hr before and after drug administration. Two litres of each pooled sample was extracted and aliquots were chromatographed as described above. The remainders of each butanol concentrate were applied to separate thin-layer plates as a number of spots along the starting line. Reference compounds were applied near both margins, and the chromatograms developed in solvent system 1 (Table 1). The plates were dried and the reference compounds located by spraying the margins of the plates with Dragendorff's reagent. The silica gel in the region between each reference compound was scraped from chromatograms of test and control urine extracts. The four silica gel samples thus obtained were placed separately into glass-stoppered centrifuge tubes. Distilled water (3 ml) and concentrated ammonia solution (0·5 ml) were added to each and the contents extracted with 3 × 2·5 ml portions of diethyl ether. The four sets of ethereal extracts were each combined, evaporated to low volume and allowed to evaporate on a potassium bromide disc. The discs were then placed into an infrared recording spectrophotometer, the urinary excretion product in the sample beam and its control in the reference beam.

Results and discussion

CHEMICAL FINDINGS

The properties of the base IV formed by reaction between 3-cyano-1-methyl-3,3-diphenylpropyl isocyanate and ethylmagnesium bromide were reported by Bretschneider & others (1959). Its structure may be represented by the tautomeric pair (IVa and IVb-flow sheet 1) as it behaves both as an exo- and endo-cyclic alkene. When treated with dimethyl sulphate it formed an *N*-methyl derivative isolated as the iodide V. This salt was identical with the hydriodide IX of the base VIII obtained from the reaction

between the pyrrolidone VI and ethyl-lithium: the hydriodide IX lacked an absorption band in the νO-H region and its melting point (151–153°) was close to that (150–152°) of the iodide V derived by the Bretschneider route. Unequivocal identity of the two iodide compounds was established by their coincident infrared spectra and undepressed mixed melting point (150–153°). The structure of the iodide V may be represented by the tautomeric pair Va and Vb (flow sheet 1). However, the infrared and nmr spectra of the iodide V were inconsistent with the exocyclic alkene structure (Vb) in the following respects:

Infrared. (i) The infrared spectrum showed no $\nu\overset{+}{N}$-H band, characteristic of tertiary amine salts. (ii) The intensity of the absorption band near 1660 cm^{-1} was unusually high for a C=C stretching frequency.

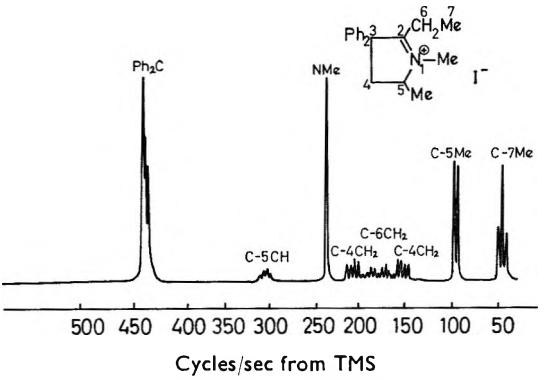


FIG. 1. 100 megacycle nmr spectrum of the pyrrolidine hydriodide (V) in $CDCl_3$

Nmr, (Fig. 1) (i) The methyl group of the C-2 substituent gave a triplet signal rather than the doublet expected for $>=CH\mathit{Me}$ in the exocyclic structure. (ii) No vinylic signal was obtained (the multiplet at 307 cycles/sec is assigned to the C-5 methine proton from spin-spin decoupling results).

If the product were an isomeric mixture of alkenes, the triplet may have resulted from the overlap of two doublets. This possibility was disproved by the fact that the same Me triplet was also clearly apparent in a spectrum recorded at 100 megacycles/sec. These anomalous spectral results may be interpreted, however, if the iodide V has the endocyclic structure (Va). In this formula the acidic proton is attached to a carbon atom rather than to the nitrogen atom and hence no $\nu\overset{+}{N}$-H band should be observed in the infrared spectrum, while the C = N (rather than C = C) function accounts for the strong band near 1660 cm^{-1}. The methyl group is adjacent to a methylene group and must give a triplet nmr signal as observed.

Further nmr evidence for the endocyclic structure was obtained by deuteration and spin-spin decoupling studies as follows: (1) On examination of the iodide V in $CDCl_3$-D_2O, the methylene proton signals of the CH_2Me group were absent and the methyl group triplet became a singlet

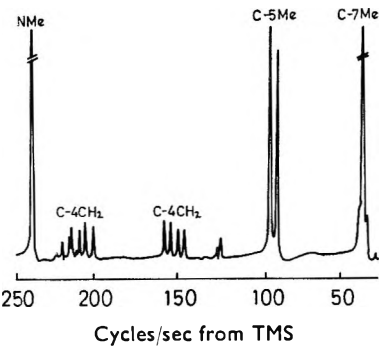


FIG. 2. 100 megacycle nmr spectrum of the pyrrolidine hydriodide (V) in $CDCl_3$-D_2O.

(Fig 2). If the methylene carbon atom is the site of protonation, the two protons attached at this point will both be acidic and capable of exchange. Hence their signal should disappear on deuteration and their coupling action with CH_2Me will be disrupted as observed. (2) On spin-spin decoupling of the methine proton of the CHMe group, the CH_2 methylene protons adjacent to phenyl group collapsed to two doublets (AB quartet) and the s-Me doublet collapsed to a singlet. (3) Spin-spin decoupling of the s-Me doublet resulted in the collapse of the methine proton signal of the CHMe group to a broad triplet. (4) Spin-spin decoupling of the methyl group triplet of the CH_2Me group, caused the methylene protons signal of the CH_2Me group to be changed from a near octet to a broad AB-type quartet. All these results are in accord with the endocyclic alkene assignment.

METABOLIC FINDINGS

The results of the preliminary thin-layer and gas-liquid chromatographic experiments (Tables 1 and 2 respectively) were identical in all four subjects. Two basic urinary excretion products of methadone were detectable in

TABLE 2. GAS-LIQUID CHROMATOGRAPHY OF METHADONE, COMPOUND V AND THE URINARY EXCRETION PRODUCTS OF METHADONE

Column	Retention time of methadone (min)	Retention time of compound V (min)	Peaks appearing in test chromatogram and absent in control chromatogram	
			Number	Retention time (min)
A	14·2	9·5	2	14·2 9·5
B	7·9	5·0	2	5·0 7·9

urine and these have the same chromatographic properties as methadone and compound V. Chemical identity was established by isolation of the basic excretion products from urine and comparison of their infrared spectra with those of methadone and compound V (Figs 3 and 4). The use of spray reagents (e.g. ferric chloride, ninhydrin and diazotised *p*-nitroaniline) with specific colour reactions for phenols and primary and

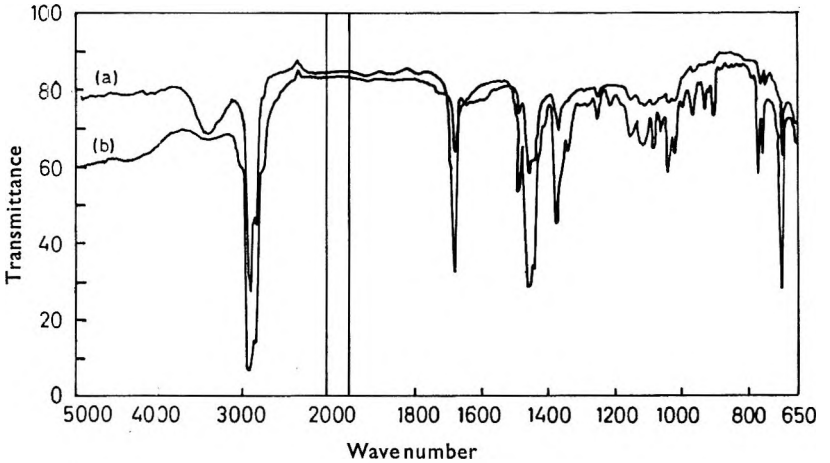


FIG. 3. The infrared spectra of methadone isolated from urine (a) and authentic methadone (b).

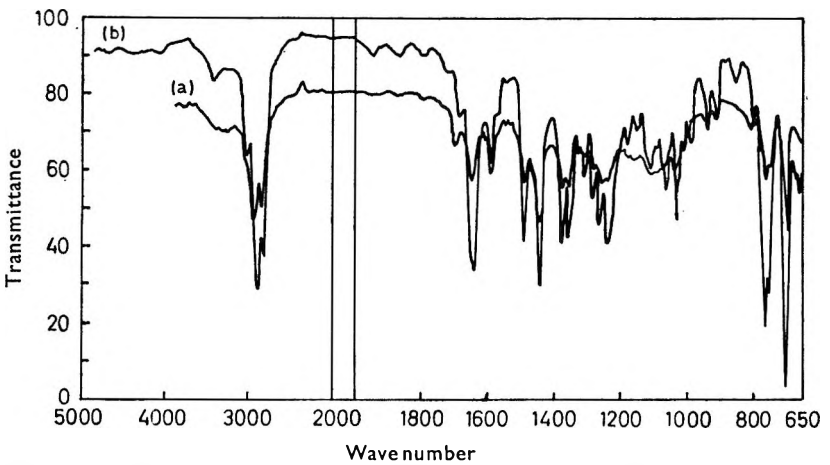


FIG. 4. The infrared spectra of a methadone metabolite isolated from urine (a) and compound V (b).

secondary amines, failed to demonstrate such compounds as excretion products of methadone. The results indicate that *N*-demethylation of methadone in man produced compound V. The chemical route of Flow Sheet 1 probably proceeds via the secondary amine 1b, formed by hydrolysis of III, and it may be postulated that the metabolic conversion of methadone to compound V involves similar *N*-demethylated intermediates.

Controlled urinary excretion experiments in man (Beckett & Taylor, unpublished data) have shown that about 60% of the methadone dose can be accounted for as unchanged and mono-*N*-demethylated drug. Therefore it is possible that for methadone or metabolite or both there are other excretory routes (e.g. via the intestines) or other pathways of metabolism.

In contrast to the findings of Vidic (1957), primary and secondary amine metabolites were not detected in the excretion products of methadone. Therefore it is unlikely that the methadone metabolite V is further *N*-demethylated.

Acknowledgements. We thank Burroughs Wellcome & Company for supplying a sample of methadone hydrochloride. One of us (J. F. T.) thanks the Science Research Council for a grant in support of this research.

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J. Pharm. Pharmac., 1968, **20**, 763–767 Received February 28, 1968

A note on the effects of 2, 4-diamino-5-phenylthiazole and 1, 2, 3, 4-tetrahydro-9-aminoacridine on morphine metabolism

J. T. C. WOO, G. A. GAFF AND M. R. FENNESSY

Chromatograms of extracts of urine from patients receiving mixtures of morphine plus amiphenazole or morphine plus 1,2,3,4-tetrahydro-9-aminoacridine (THA) have a spot which is due to a substance with properties similar to morphine-*N*-oxide. This spot was not present in urine of patients given morphine, amiphenazole or THA alone. Morphine-*N*-oxide may be a metabolite of morphine, and amiphenazole and THA may inhibit the further metabolism of morphine-*N*-oxide.

THE use of combinations of 2,4-diamino-5-phenylthiazole (amiphenazole) and morphine, or 1,2,3,4-tetrahydro-9-aminoacridine (THA) and morphine for the relief of chronic pain has been reported to have advantages over treatment with morphine alone. In patients with intractable pain from terminal carcinoma given morphine and amiphenazole there was no narcosis, and the respiratory depressant action of morphine was absent (Shaw & Shulman, 1955, 1955a). McKeogh & Shaw (1956) confirmed these observations and showed that, with this drug combination, analgesia was not impaired. Marked tolerance to morphine was not observed, and when this drug regimen was withdrawn there was no evidence of an abstinence syndrome. Similar effects were reported when THA was administered concurrently with morphine (Shaw, 1960; Stone, Moon & Shaw, 1961). These advantages were attributed by Gershon, Bruce & others (1958) to the *in vivo* formation of a "complex" between morphine and amiphenazole. The present work deals with an attempt to detect and identify such a substance in the urine of cancer patients given morphine plus amiphenazole, or morphine plus THA, or amiphenazole, THA or morphine alone.

Experimental

Examination of urine samples. Urine samples were collected over a 24 hr period from cancer patients (Austin Hospital, Heidelberg, Victoria) receiving morphine, THA or amiphenazole, or mixtures of amiphenazole and morphine or of THA and morphine; other drugs were given in addition to the above as demanded by the needs of the patients.

The urine sample (250 ml) was adjusted to pH 9·0 with 50% NaOH and shaken with an equal volume of an isopropanol–chloroform (1 : 3) mixture for 10 min. Two layers slowly formed and the separated organic phase was centrifuged at 2,800 rev/min for 10 min. This phase was reduced under low pressure to <2 ml, and then made up to 2 ml with the isopropanol–chloroform mixture.

Paper chromatography. The concentrated organic extract (30 μl) was spotted on Whatman No. 1 paper and thoroughly dried. Solutions containing 25 μg each of amiphenazole, THA, morphine, amiphenazole plus morphine, and THA plus morphine were used as markers. Ascending

From the Department of Pharmacology, University of Melbourne, Parkville, Victoria 3052, Australia.

chromatography was carried out at 20°. The solvent systems were n-butanol–ammonia (sp. gr. = 0·88)–water (4:1:3, v/v), n-butanol–pyridine–water (6:4:3), n-butanol–methyl isobutyl ketone–33% ethylamine (2:2:1) and n-butanol–isobutyric acid–water (7:2:3). The detecting agents were diazotized *p*-aminoacetophenone (Lundgren, 1956), iodoplatinate and bromcresol green (Smith, 1960).

Synthesis of a substance formed by the interaction of morphine and amiphenazole. This was as reported by Woo (1965) and involves mixing solutions of morphine and amiphenazole in methanol, adding concentrated ammonia and allowing the mixture to react for 15 hr at room temperature (20°). Unreacted morphine and amiphenazole are then removed from the mixture after drying by extraction with ethyl acetate under alkaline conditions. The product is purified using column chromatography and by recrystallization.

Spectroscopy. Infrared spectra of solid samples were measured, using paraffin oil mulls, with a Perkin-Elmer infrared spectrophotometer (model 137). Ultraviolet spectra were measured with a Beckman spectrophotometer DU (model G2400).

Elemental analysis for C, H, N and O were performed by the Australian Micro-analytical Service (C.S.I.R.O.).

Morphine-*N*-oxide (m.p. 272–273°) was synthesized using the method of Freund & Speyer (1910, m.p. 273°).

Results

Chromatograms of extracts of the urine of 5–10 patients receiving either morphine and amiphenazole or morphine and THA showed a spot not found in the urine of patients receiving morphine, amiphenazole or THA alone nor was it present in urine from patients who were not receiving drugs. The Rf values of the unknown substance and its colour reactions with various detecting agents were identical to those of authentic morphine *N*-oxide (Table 1). But we have not, as yet, isolated sufficient to make a positive identification.

TABLE 1. Rf VALUES AND COLOUR REACTIONS WITH DETECTING AGENTS

Solvent system	Morphine	Amiphenazole	THA	Substance in urine	Substance synthesized	Morphine *N*-oxide
			Rf values			
n-Butanol–ammonia–water	0·70	0·88	0·90	0·15	0·15	0·14
n-Butanol–pyridine–water	0·31	0·87	0·55	0·55	0·55	0·53
n-Butanol–methylisobutyl ketone–ethylamine ..	0·45	—	0·85	0·00	0·00	0·00
n-Butanol–isobutyric acid–water	0·48	0·56	0·73	0·62	0·62	0·64
Reagent			Colour of chromatograph spots			
Diazotized *p*-amino acetophenone	Purple	Yellow-orange	Nil	Deep-purple	Deep-purple	Deep-purple
Iodoplatinate	Purple-blue-black	Nil	Violet	Violet-purple	Violet-purple	Violet-purple
Bromcresol green ..	Blue	Nil	Powder-blue	Purple-blue	Purple-blue	Purple-blue

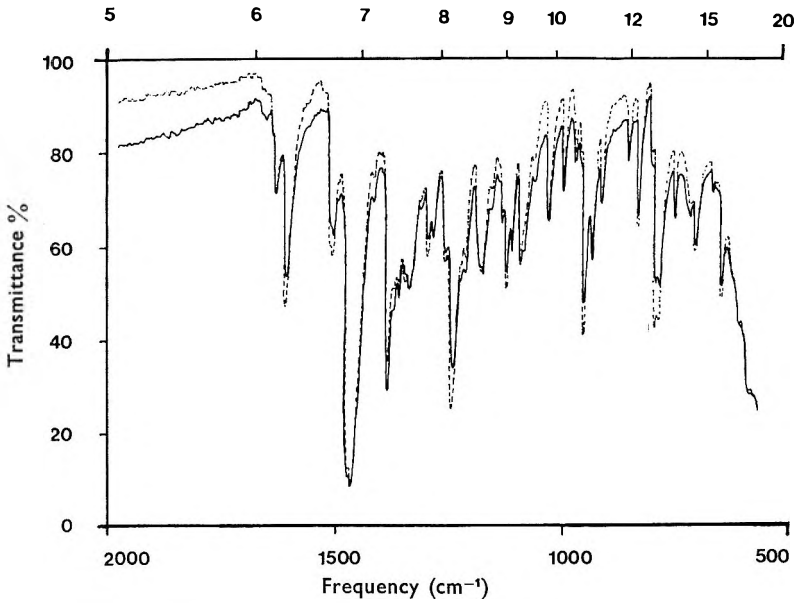

FIG. 1. Infrared spectra of morphine-*N*-oxide (broken line) and the substance synthesized by mixing morphine and amiphenzaole (solid line).

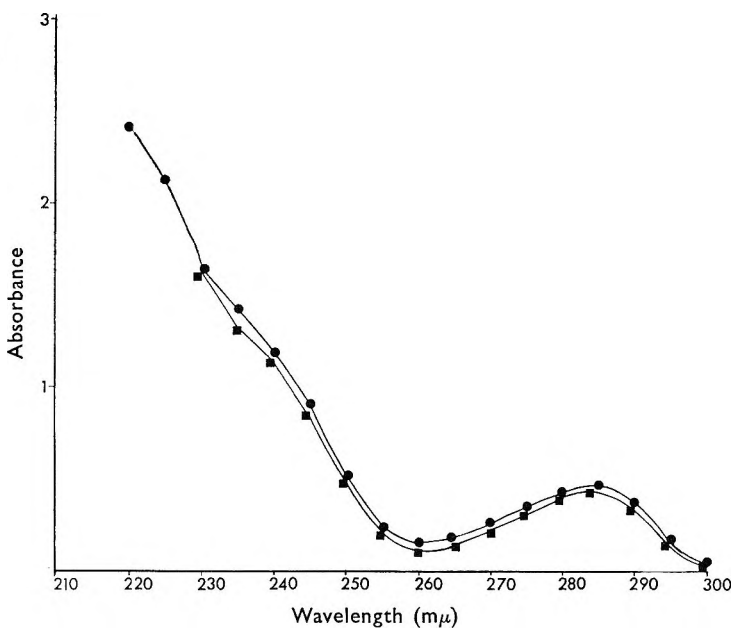

FIG. 2. Ultraviolet spectra of aqueous solutions of morphine-*N*-oxide (●——●) and the substance synthesized by mixing morphine and amiphenazole (■——■).

The substance synthesized from the mixture of morphine and amiphenazole was a white powder, m.p. 251–256°. Elemental analysis of three samples (mean found: C, 66·7; H, 6·3; N, 4·7%) showed that the empirical formula ($C_{16·5}$,$H_{18·6}$,N, O_4) corresponded closely to that of morphine *N*-oxide (C_{17},H_{19},N, O_4) suggesting that the substance synthesized was morphine *N*-oxide, and this was further confirmed by the similarity of the colour reactions and chromatographic properties (Table 1) in comparison with the authentic morphine *N*-oxide, and of the close similarity of their infrared (Fig. 1) and ultraviolet spectra (Fig. 2).

Discussion

The substance synthesized by reacting together morphine and amiphenazole would seem to be morphine *N*-oxide on the evidence of the infrared and ultraviolet spectra, elemental composition and paper chromatography. Because of the similarity in chromatographic properties of the synthetic substance and the substance detected in urine, it is possible that morphine *N*-oxide is a metabolite of morphine. Several drugs are already known to be excreted to some extent by man, as *N*-oxides, notably chlorpromazine (Fishman, Heaton & Goldenberg, 1962), imipramine (Fishman & Goldenberg, 1962) and chlorcyclizine (Kuntzman, Phillips & others, 1967). Also, studies with animals have shown that other drugs undergo *N*-oxidation and that NADPH- and oxygen-dependent liver microsomal enzymes are capable of forming *N*-oxides *in vitro* (McMahon, 1966).

A generally recognized metabolic fate of morphine is demethylation. The evidence pointing to the metabolic formation of morphine *N*-oxide from morphine is of interest because of the hypothesis originally suggested by Fish, Johnson & others (1955) that *N*-oxides are intermediates in the oxidative demethylation of drugs.

The substance excreted in the urine was detected only when either THA or amiphenazole was administered in conjunction with morphine but not when morphine, THA or amiphenazole were given alone. This suggests that THA and amiphenazole cause excretion of morphine *N*-oxide either by inhibiting its degradation to other metabolites or by inhibiting an alternative pathway for the metabolism of morphine. Less likely is the possibility that THA and amiphenazole stimulate the formation of morphine *N*-oxide directly. As the *N*-oxide was not found in the urine of patients treated with morphine alone it seems unlikely that its formation could be due to an artefact arising from the extraction procedure or by bacterial action.

The hypothesis of Gershon & others (1958) that combined administration of morphine with amiphenazole or THA leads to formation of a complex between morphine and those drugs would not appear to be substantiated.

Acknowledgements. We wish to thank Professor F. H. Shaw for his supervision of part of this work. Thanks are also due to Dr. W. Moon of the Austin Hospital, Melbourne, for his cooperation and to H. W. Woods Pty. Ltd. for financial assistance. The authors acknowledge Professor M. J. Rand for his criticism and revision of the typescript.

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J. Pharm. Pharmac., 1968, **20**, 768–774 Received February 13, 1968

1-Allyl and 1-(3,3-dimethylallyl) analogues of pethidine and its reversed ester

A. F. CASY*, A. B. SIMMONDS AND D. STANIFORTH

The preparation of some 1-allyl and 1-(3,3-dimethylallyl)-4-phenylpiperidines related to pethidine and its reversed ester is described and the hot-plate activities in mice of these compounds reported. All six derivatives displayed morphine-like properties in mice, four being more potent (1·5–6 times) than morphine, but failed to act as analgesic antagonists in rats. The results are contrasted with the properties of similarly *N*-substituted fused-ring analgesics and differences discussed in terms of drug-receptor interaction modes.

THE aim of this work was to investigate the effect of replacing the methyl group in position 1 of 4-phenylpiperidine analgesics, such as pethidine and its reversed ester, by an allyl or 3,3-dimethylallyl group. In morphine, levorphanol and metazocine (analgesics with fused-ring skeletons), the same structural modification causes a striking change in pharmacological properties, the *N*-allyl (and *N*-substituted allyl) derivatives being analgesic antagonists (the *N*-allyl compounds have a potent action and the *N*-3,3-dimethylallyl compounds a weak action) which are devoid of activity in most of the usual tests for analgesia in animals. In man, however, some of these derivatives are effective analgesics, and one of them, pentazocine, has been developed as a clinically useful non-addicting analgesic (Keats & Telford, 1964; Archer & Harris, 1965).

Chemistry

The pethidine analogue I was obtained by alkylating norpethidine with 1-chloro-3-methylbut-2-ene. The 1-allyl-4-piperidones II (R = H or Me) required for the synthesis of the reversed esters III were made by Dieckmann cyclizations of the appropriate acyclic amino-diesters. Treatment of these piperidones with phenyl-lithium followed by propionic anhydride gave the esters III. In the case of the 3-methyl derivative III (R = Me), only one of the two diastereoisomeric esters was isolated and this was assigned a *trans* 3-Me/4-Ph configuration from a consideration of the nmr data (the secondary Me chemical shift of this ester suffered only a minor downfield shift upon base protonation) (Casy, 1966). The 1-(3,3-dimethylallyl) derivatives IV were obtained by alkylating the corresponding 4-phenyl-4-piperidinols with 1-chloro-3-methylbut-2-ene (or acylating with 3,3-dimethylacryloyl chloride and then reducing the amide formed) and esterifying the resulting piperidinol with propionic anhydride.

From the Department of Pharmacy, Chelsea College of Science and Technology (University of London), London, S.W.3, England.

* Present address: Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada.

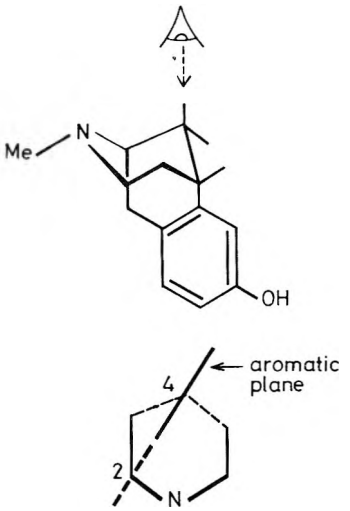
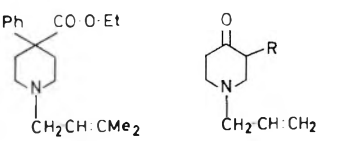
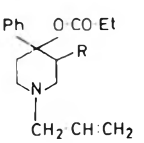


Diagram of the 4-phenylpiperidine moiety of fused-ring analgesics. In the lower drawing the piperidine and aromatic rings are depicted as viewed from above the piperidine and aromatic rings by an observer whose eye is placed in the position shown.

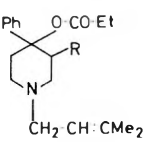


I

II

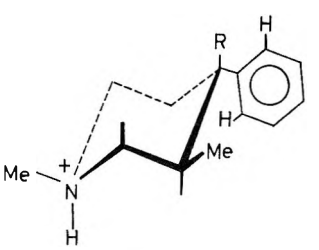


III

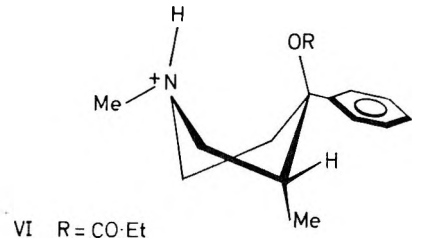


IV

R = H or Me



V R=O·CO·Et

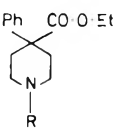
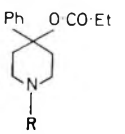
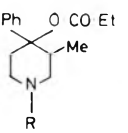


VI R = CO·Et

Pharmacology and discussion

The analgesic activity of the pethidine congener I and the reversed esters III and IV (R = H or Me) was assessed in mice by the hot-plate

TABLE 1. HOT-PLATE ACTIVITIES OF SOME 1-ALLYL- AND 1-(3,3-DIMETHYLALLYL)-4-PHENYLPIPERIDINES IN MICE*

Structure	ED50 mg/kg	
	R = CH_2·CH = CH_2	R = CH_2·CH = CMe_2
	63†	40
	15	7·5
	7·5‡	32

* Determined after subcutaneous administration by Janssen & Jageneau's method (1957)—by this procedure ED50 values for morphine and pethidine are 11 and 23 mg/kg respectively.
† Janssen (personal communication).
‡ ED50 for alphaprodine is 5·5 mg/kg approx. (Beckett, Casy & others, 1957).

test (Table 1). A result for the *N*-allyl analogue of pethidine, a compound previously reported to be without effect upon analgesia produced by methadone, alphaprodine and pethidine as assessed by a test in rats (Costa & Bonnycastle, 1955) is included. Nalorphine-like antagonistic properties were evaluated in rats using morphine (40 mg/kg subcutaneously) as the reference agonist. The test compound, given intravenously 1 hr after morphine, was considered to have nalorphine-like activity only if the morphine-induced rigidity and inhibition of the corneal and pinnal reflexes were antagonized immediately. By this criterion, all the

compounds tested were devoid of nalorphine-like properties. Thanks are due to Dr. Paul Janssen for making these tests.

The six compounds listed in Table 1 displayed morphine-like properties in mice, as shown by their mydriatic and behavioural effects (excitation and Straub tail) and significant hot-plate ED50 values (some of the compounds were more potent than morphine) but failed to act as analgesic antagonists in rats. The *N*-3,3-dimethylallyl analogue of ketobemidone is also a significantly active analgesic in mice (hot-plate ED50 = 4·6, cf. ketobemidone ED50 = 1·6) while it fails to display typical nalorphine-like effects in normal monkeys (Dr. J. E. Villarreal, personal communication). In 1-methyl-4-phenylpiperidine compounds with analgesic activity, therefore, no radical pharmacological change results when the methyl group in position 1 is replaced by allyl functions, a result in marked contrast to findings relating to cyclic analgesics of greater molecular rigidity such as morphine.

Although it is generally true that *N*-substituents have similar influences upon analgesic activity in the two classes of compounds (e.g. the potency-raising effect of the *N*-phenethyl group), discrepancies have been reported. Thus, while replacement of the methyl group in position 1 by a cinnamyl or 3-phenylpropyl group gives potent pethidine analogues (Elpern, Carabateus & others, 1959; Elpern, Gardner & others, 1957), the corresponding normorphinan derivatives are inactive (Eddy, Besendorf & Pellmont, 1958). Conversely, although *N*-phenacylnormorphinan is 6·5 times as potent as levorphanol, the same norpethidine derivative is only one tenth as active as the parent 1-methyl compound (Janssen & Eddy, 1960). The two classes also show different structure-activity relations with regard to oxygen functions. In analgesics based on morphine, morphinan and benzomorphan, a free phenolic group is an important, and often essential, feature for activity (its removal or etherification* result in sharp falls in potency) but the same function is not a prerequisite for high potency in 4-phenylpiperidine analgesics, although it may be advantageous (e.g. ketobemidone); on the other hand, all potent analgesics of the latter class possess non-aromatic oxygenated functions (e.g. CO·O·Et, O·CO·Et) (Beckett & Casy, 1965 and refs. there cited).

While both analgesic types appear to interact at a common site (evidence from analgesic antagonists), their differing structure-activity relations, as outlined above, indicate that they probably vary in their association modes with the receptor. Similar conclusions have been reached for two classes of acyclic analgesics, represented by methadone and diampromid respectively, which differ both in basic-group structure and stereospecificity (Casy & Hassan, 1967). The 4-phenylpiperidine unit is common to simple piperidine derivatives such as pethidine and alphaprodine and to morphine, morphinan, and benzomorphan analgesics. This fact allows a superficial correlation between rigid and non-rigid

* Esterification often leads to an enhancement of potency, but a study of diamorphine (heroin) indicates that the pharmacological effects of such esters are mediated primarily by the free phenols (Way, Kemp & others, 1960; Way, 1967).

cyclic analgesics but should not be interpreted too narrowly in terms of molecular geometry. In rigid analgesics the 4-phenylpiperidine moiety is constrained to an axial-phenyl chair conformation with the aromatic plane parallel with one passing through a line joining C–2 and C–4 of the heterocyclic ring (Diagram). In simple 4-phenylpiperidines, however, likely conformations for pethidine and alphaprodine (V) and for betaprodine (VI) as solutes in water have been proposed (Casy, 1966, 1968) in which the piperidine–aromatic ring orientations (deemed a key factor in agonist–receptor association, as this unit contains both the electrostatic and van der Waals' binding centres) differ markedly from that shown in the diagram. Thus, if flexible piperidine analgesics are to present the orientation shown in the diagram to the receptor, they must adopt highly unfavoured conformations, and it seems more reasonable to postulate a receptor capable of adapting itself to a variety of agonist conformations of varying binding efficiencies.

Experimental

1-*Allyl*-4-*piperidone*. A mixture of allylamine (19 g), ethyl acrylate (36·5 g) and ethanol (500 ml) was left to stand for two days, and then fractionally distilled to give ethyl β-(allylamino)-propionate (79 g), b.p. 66°/1·6 mm (Speziale & Jaworski, 1960, give b.p. 56°/1·2 mm). It formed a *hydrogen oxalate*, m.p. 184–6°, from acetone-ether (Found: C, 48·3; H, 7·0; N, 5·6. $C_{10}H_{17}NO_6$ requires: C, 48·6; H, 6·9; N, 5·7%). The aminoester (35 g) and ethyl acrylate (24·5 g) in ethanol (250 ml) were heated under reflux for 12 hr, and then fractionally distilled to give *N*-allyldi(2-ethoxycarbonylethyl)amine (48 g), b.p. 112–4°/2 mm. It formed a *hydrogen oxalate*, m.p. 87°, from acetone-ether (Found: C, 51·7; H, 7·2; N, 4·3. $C_{15}H_{25}NO_8$ requires: C, 51·9; H, 7·2; N, 4·0). The tertiary amine diester (103 g) was cyclized with sodium hydride (20·5 g) in benzene (2 litres) containing ethanol (1 ml) and then decarboxylated with HCl by the usual procedures (Casy, Birnbaum & others, 1965) to give *1-allyl-4-piperidone* (39·5 g), b.p. 60–63°/3 mm, n_D^{19} 1·4783 (Found: C, 68·7; H, 9·3; N, 9·7; $C_8H_{13}NO$ requires: C, 69·0; H, 9·4; N, 10·1%).

1-*Allyl*-3-*methyl*-4-*piperidone*. A mixture of allylamine (19 g), methyl methacrylate (36·7 g) and ethanol (100 ml) was stirred for 6 hr, and then heated under reflux for 16 hr. The product was fractionally distilled to give *methyl β-allylamino-α-methylpropionate* (30 g), b.p. 74°/0·65 mm (Found: C, 61·5; H, 9·9; N, 8·8. $C_8H_{15}NO_2$ requires: C, 61·1; H, 9·6; N, 8·9%). The amino-ester (42 g) and ethyl acrylate (29·4 g) in ethanol (150 ml) were heated under reflux for 24 hr and fractionally distilled to give *N*-allyl-*N*-(2-ethoxycarbonylethyl) (2-methoxycarbonylpropyl)amine (61·5 g), b.p. 104–116°/0·35 mm, n_D^{20} 1·4512. It gave an *oxalate*, m.p. 176°, from acetone-ether (Found: C, 55·6; H, 9·0. $C_{28}H_{48}N_2O_{12}$ requires: C, 55·6; H, 9·4%). The tertiary amine diester (78·5 g) was cyclized and decarboxylated as before to give 1-allyl-3-methyl-4-piperidone (28 g), b.p. 65°/0·4 mm (Found: C, 69·8; H, 9·65. $C_9H_{15}NO$ requires:

C, 70·55; H, 9·9%). It gave a *hydrobromide*, m.p. 141° (Found: C, 46·0; H, 6·7; N, 6·1. $C_9H_{16}BrNO$ requires: C, 46·2; H, 6·9; N, 6·0%).

1-*Allyl*-4-*phenyl*-4-*propionyloxypiperidine and its* 3-*methyl analogue.* 1-Allyl-4-piperidone (14 g) in ether (100 ml) was added to a cooled ethereal solution of phenyl-lithium prepared from lithium (4·4 g) and bromobenzene (50·2 g), the mixture heated under reflux for 2 hr, and then treated with propionic anhydride (91 g). After stirring for 16 hr, the mixture was poured onto ice and aqueous NaOH, and the ethereal phase separated and extracted with aqueous HCl. The base recovered from this extract was distilled to give 1-allyl-4-phenyl-4-propionyloxypiperidine (16 g), b.p. 179/1 mm. It gave a *hydrobromide*, m.p. 193°, from isopropanol (Found: C, 57·8; H, 6·7; N, 4·1. $C_{17}H_{24}BrNO_2$ requires: C, 57·6; H, 6·5; N, 4·0%) and a *toluene*-p-*sulphonate*, m.p. 140°, from ethyl acetate (Found: C, 64·1; H, 6·9; N, 3·1. $C_{24}H_{31}NO_5S$ requires: C, 64·7; H, 7·0; N, 3·1%). Similar treatment of 1-allyl-3-methyl-4-piperidone (10 g) gave 1-allyl-3-methyl-4-phenyl-4-propionyloxypiperidine (11 g), b.p. 148°/0·7 mm. It gave a *hydrobromide*, m.p. 210°, from isopropanol (Found: C, 58·9; H, 7·0; N, 3·8. $C_{18}H_{26}BrNO_2$ requires: C, 58·7; H, 6·8; N, 3·8%).

1-(3,3-*Dimethylallyl*)*norpethidine.* Norpethidine (10 g) and 1-chloro-3-methylbut-2-ene were dissolved in acetone (200 ml) through which CO_2-free nitrogen had been passed, and the mixture was stirred at the reflux temperature with Na_2CO_3 (13·6 g) for 48 hr. The filtered mixture was evaporated and the residue distilled to give 1-(3,3-dimethylallyl)-norpethidine (8 g), b.p. 170–172°/2 mm. It gave a *hydrobromide*, m.p. 199°, from isopropanol (Found: C, 59·7; H, 7·4; N, 3·5. $C_{19}H_{28}BrNO_2$ requires: C, 59·7; H, 7·4; N, 3·7%).

1-(3,3-*Dimethylallyl*)-4-*phenyl*-4-*propionyloxypiperidine and its* 3-*methyl analogue.* 1-Chloro-3-methylbut-2-ene (5·8 g) was added dropwise to a stirred mixture of 4-phenyl-4-piperidinol (9 g), benzene (150 ml), acetone (100 ml) and Na_2CO_3 (16 g). The mixture, after heating under reflux for 6 hr, was filtered and the filtrate fractionally distilled to give 1-(3,3-dimethylallyl)-4-phenyl-4-piperidinol (7 g), b.p. 142°/0·9 mm. This alcohol (6 g) was also obtained by reducing 1-($\beta\beta$-dimethylacryloyl)-4-phenyl-4-piperidinol (10 g) with aluminium lithium hydride (1·5 g) in tetrahydrofuran (150 ml); this *amide* (6 g), m.p. 104°, from chloroform (Found: C, 73·7; H, 7·95; N, 5·1. $C_{16}H_{21}NO_2$ requires: C, 74·1; H, 8·1; N, 5·4%) was obtained by treating 4-phenyl-4-piperidinol (8 g) with $\beta\beta$-dimethylacryloyl chloride (5·3 g) (Smith & Englehart, 1949) in the presence of Na_2CO_3 (10 g). A mixture of 1-(3,3-dimethylallyl)-4-phenyl-4-piperidinol (2 g), pyridine (3 ml) and propionic anhydride (3 ml) was heated under reflux for 3 hr and the product then decolorized with charcoal, filtered and evaporated. The residue, neutralized with 10% HBr in isopropanol and diluted with ether, gave 1-(3,3-*dimethylallyl*)-4-*phenyl*-4-*propionyloxypiperidine hydrobromide* (1·6 g), m.p. 181°, from isopropanol (Found: C, 60·0; H, 7·2; N, 3·9. $C_{19}H_{28}BrNO_2$ requires C, 59·7; H, 7·4; N, 3·7%). 3-Methyl-4-phenyl-4-piperidinol (15 g) Carabateas & others, 1963) treated with 1-chloro-3-methylbut-2-ene (9 g)

and $NaHCO_3$ (20 g), as previously described, gave 1-(3,3-*dimethylallyl*)-3-*methyl*-4-*phenyl*-4-*piperidinol* (6 g), b.p. 180–190°/1·5 mm (Found: C, 78·8; H, 9·3; N, 5·5; equiv. wt 261. $C_{17}H_{25}NO$ requires: C, 78·7; H, 9·7; N, 5·4%; equiv. wt 259). This alcohol (3 g), esterified with propionic anhydride (5 ml) and pyridine (5 ml), as previously described, gave 1-(3,3-*dimethylallyl*)- 3 -*methyl*- 4 -*phenyl*- 4 -*propionyloxypiperidine hydrobromide*, m.p. 169°, from isopropanol (Found: N, 3·7. $C_{20}H_{30}BrNO_2$ requires: N, 3·5%). Nmr characteristics of the 3-methyl substituent: Base, doublet 40 cycles/sec (J 6·7 cycles/sec); HBr, doublet 42 cycles/sec (J 6 cycles/sec) from tetramethylsilane.

Nmr spectra were recorded at 60 megacycles/sec on a Perkin-Elmer R–10 instrument, using $CDCl_3$ as solvent and tetramethylsilane as internal standard.

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The chromatographic analysis of spermaceti

P. J. HOLLOWAY*

The alkyl esters of spermaceti can be analysed directly by high temperature gas chromatography on a 10% Silicone Elastomer E301 column following the injection of the whole wax. The validity of the technique was established by initial chromatographic work on the isolated alkyl ester fraction and its saponification products. In three commercial wax samples, four homologues C_{28} (hexadecyl dodecanoate), C_{30} (hexadecyl tetradecanoate), C_{32} (hexadecyl hexadecanoate) and C_{34} (hexadecyl octadecanoate), accounted for 86–89% of the total alkyl esters. Variation in the ester homologue content is produced only by variations in the fatty acid homologue content.

THE major constituents of spermaceti have long been known to be long chain alkyl esters comprised of primary alcohols and saturated fatty acids. However, there is no general agreement about the exact composition of the ester fraction. Most authors report that the fraction is chiefly cetyl palmitate $C_{15}H_{31}COOC_{16}H_{33}$ (Pratt & Youngken, 1956; Trease, 1966; Claus & Tyler, 1965; Warth, 1956). Others have reported the esters to be predominantly a mixture of cetyl palmitate and cetyl myristate in approximately equal proportions (Wallis, 1962; BPC, 1963).

This paper describes further work on the alkyl ester constituents of spermaceti using thin-layer, preparative-layer and gas chromatographic methods for analysis.

Experimental

Melting points were determined on a Kofler block. Infrared absorption spectra were obtained with a Unicam SP 200 infrared spectrophotometer; the samples were examined in potassium chloride discs (1–2 mg in 200 mg).

THIN-LAYER CHROMATOGRAPHY

The qualitative composition of the wax was determined by the methods of Holloway & Challen (1966). Wax constituents are fractionated into classes of compound, not into individual compounds, by thin-layer chromatography (Malins & Mangold, 1960). The alkyl ester content of the wax was determined by preparative-layer chromatography on 1·5 mm layers of Kieselgel H using the solvent system carbon tetrachloride/chloroform: 95/5 v/v (Holloway 1967). The alkyl ester fraction isolated was identified by infrared spectroscopy, the ferric hydroxamate colour test (Goddu, Leblanc & Wright, 1955) and further thin-layer tests (Holloway & Challen, 1966).

GAS CHROMATOGRAPHY

A Pye Series 104 gas chromatograph fitted with a flame ionization detector, and a −0·1 to + 1·0 mV Honeywell-Brown Electronik recorder were used. The chromatographic columns were of stainless steel 4 mm in bore and 5 ft in length, packed with 100–120 mesh Chromosorb W coated

From the School of Pharmacy, University of London, Brunswick Square, London, W.C.1.

* Present address: Long Ashton Research Station, University of Bristol, Bristol, England.

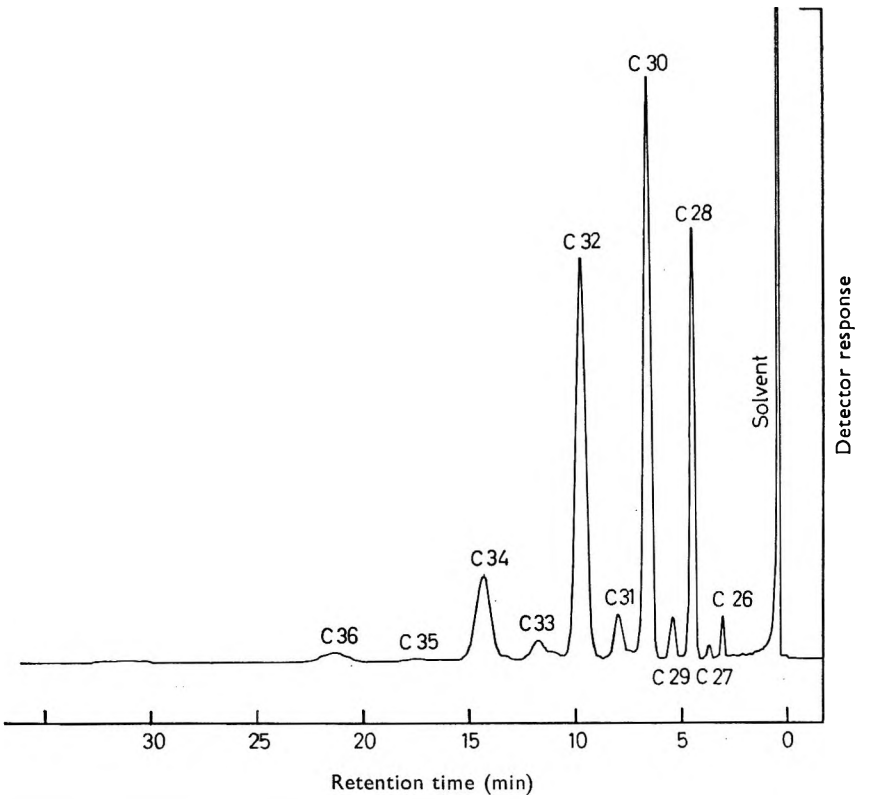

FIG. 1. Gas chromatogram of the analysis of the alkyl ester fraction from spermaceti in Sample 2.

with 10% w/w Silicone Elastomer E301 (ICI Ltd). The carrier gas was nitrogen, flow rate 40 ml/min (18 lb/inch^2), and the detector flow rates were hydrogen 40 ml/min (15 lb/inch^2) and air 420 ml/min (20 lb/inch^2). Chromatograms were run isothermally using a chart speed of 20 inches/hr. All samples were injected as 2·0% solutions in chloroform or benzene; the injection volume being in the range 0·2–1 μl. Before use the columns were conditioned for at least 24 hr with the oven temperature 25° above the required operating temperature and using full carrier gas flow.

Spermaceti and its alkyl ester fraction were analysed at 300°. Peaks were identified by comparison of retention times with those of reference samples of alkyl esters (C_{26}, C_{28}, C_{30}, C_{32}, C_{34} and C_{40}) prepared by the method of Kaufmann & Pollerberg (1962). Saponification of the alkyl ester fraction and the recovery of the resultant alcohols and acids, were according to Mazliak (1963). The primary alcohols and fatty acids obtained were analysed isothermally at 200°. Primary alcohols were chromatographed as their acetate derivatives (Holloway, 1967) and identified by comparison with reference primary alcohol acetates (C_{12}, C_{14}, C_{16}, C_{18} and C_{20}). Fatty acids were chromatographed as their methyl

ester derivatives (Metcalfe & Schmitz, 1961) and identified by comparison with reference fatty acid methyl esters (C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, C_{20},C_{22} and C_{24}). Further identification of alkyl ester, primary alcohol acetates and fatty acid methyl esters was achieved by plotting log retention time (from the solvent peak) against carbon number (James & Martin, 1952). Completeness of elution of all chromatograms was checked by running the fractions at higher temperatures and for longer times, which would indicate the presence of any higher homologues.

The quantitative composition of the fractions was determined from the chart by computation from the peak areas using the triangulation method. The area obtained is 97% of the actual area (Condal-Bosch, 1964). Each constituent of a fraction was expressed as the ratio of its peak area to the summation of the areas of all peaks. Mean values were calculated from five replicate determinations.

Results and discussion

The major constituent of spermaceti by thin-layer chromatography corresponded in Rf value with an alkyl ester. Trace amounts of other constituents corresponding to alkane, secondary alcohol and fatty acid were also detected. No sterols or sterol esters, reported to be present in spermaceti by Warth (1956), were detected. The three commercial spermaceti samples gave identical thin-layer patterns. Adulterants of spermaceti reported by Wallis (1962) and BPC 1963 could be also readily detected by the presence of anomalous spots in the thin-layer chromatographic patterns. Paraffin wax moved above, while stearin and tallow (predominantly triglycerides) and stearic acid moved well below, the major alkyl ester spot of spermaceti.

The fraction which corresponded in Rf value with an alkyl ester was isolated by preparative layer chromatography and gave a strong violet colour with the ferric hydroxamate test. Its infrared spectrum showed strong bands at 2851–2890 cm^{-1} and medium bands at 1465–1470 cm^{-1} due to CH_3 and $-CH_2-$ absorption and medium bands at 720–729 cm^{-1} due to $-(CH_2)_4-$ absorption. Strong bands at 1730–1735 cm^{-1} and 1180 cm^{-1} were also present, due to $>C=0$ absorption. These absorptions are typical of a long-chain alkyl ester and were identical with a reference spectrum of octadecyl docosanoate. The melting points of the three fractions isolated from three samples of spermaceti were: for Sample 1, 50–51°, for Sample 2, 49–50° and for Sample 3, 48·5–49·0° (reported melting points for cetyl palmitate range from 51·5–53°). The alkyl ester content of the three waxes, as determined by preparative-layer chromatography of known weights of wax, was for Sample 1, 92·8%, for Sample 2, 95·0% and for Sample 3, 94·2%. These are the mean values based on 3 separate determinations. The figures are in agreement with Warth (1956) and Cole & Brown (1960) but higher than the 80% quoted by Wallis (1962).

Well resolved symmetrical peaks suitable for quantitative evaluation were obtained from all the fractions examined by gas chromatography.

The direct analysis of alkyl esters derived from natural waxes has only been briefly reported before by Radler (1965) and Ludwig (1966). Every peak in the three alkyl ester fractions examined corresponded in retention time with that of a n-alkyl ester. A representative gas chromatogram of the analysis of the alkyl ester fraction from spermaceti Sample 2 is shown in Fig. 1. The percentage composition of the alkyl ester fractions is recorded in Table 1. Homologues with an even number of carbon atoms predominated. The principal homologues were C_{28}, C_{30}, C_{32} and C_{34} comprising 88·7% of the total alkyl esters of sample 1, 88·8% of Sample 2 and 86·6% of Sample 3. However, the major homologues were C_{30} and C_{32}: 68·2% of Sample 1, 62·5% of Sample 2, and 59·4% of Sample 3.

TABLE 1. COMPOSITION (%) OF THE ALKYL ESTER FRACTIONS FROM THREE COMMERCIAL SPERMACETI SAMPLES

Carbon number	Sample 1*	Sample 2**	Sample 3***
<26	Trace	Trace	Trace
26		1·5	1·9
27		0·6	1·0
28	3·0	15·3	18·9
29	0·8	2·1	2·4
30	26·0	31·8	36·4
31	3·2	2·8	2·5
32	42·2	30·7	23·0
33	3·0	1·6	1·9
34	17·5	11·0	8·3
35	1·2	0·6	0·7
36	3·1	2·1	3·0
37	Trace	—	—
38	Trace	Trace	Trace
>38	—	—	—

* Sample 1—Museum sample (Pharmacognosy Dept.) M.p. 46·0–47·0°C.
** Sample 2—Supplied Brohme & Schimmer Ltd. (1965) M.p. 47·0–47·5°C.
*** Sample 3—Supplied Brohme & Schimmer Ltd. (1966) M.p. 46·0–47·0°C.

TABLE 2. COMPOSITION (%) OF THE FATTY ACIDS DERIVED FROM THE SAPONIFICATION OF THE ALKYL ESTER FRACTIONS FROM THREE COMMERCIAL SPERMACETI SAMPLES

Carbon number	Sample 1	Sample 2	Sample 3
<10	Trace	Trace	Trace
10		1·8	2·0
11		0·5	1·1
12	2·3	14·1	20·0
13	1·0	2·0	2·4
14	26·0	32·0	35·0
15	4·1	2·6	2·3
16	40·3	30·0	23·9
17	3·5	2·1	1·6
18	18·0	11·4	9·4
19	1·9	0·8	0·3
20	2·9	2·7	2·0
21	—	—	—
22	Trace	Trace	Trace
>22	—	—	—

The direct analysis of the esters, however, gives only the carbon number of the homologues but no information of the acids or alcohols which constitute the esters. The latter information was obtained from the gas

chromatographic examination of the saponification products of the alkyl ester fractions. The alcohol portion of the three esters was found to be nearly pure hexadecanol containing trace amounts of tetradecanol and dodecanol. The alkyl esters of spermaceti must therefore be predominantly hexadecyl esters. Wellendorf (1963) found that the alcohol fraction from one sample of spermaceti comprised 80% hexadecanol, with tetradecanol and octadecanol comprising the remainder. The acid portion of the esters showed a much wider distribution of homologues which satisfactorily accounted for the homologue variation of the alkyl esters (Table 2). The percentage composition of the fatty acids, although of different chain length, was similar to the alkyl esters (compare Tables 1 and 2). The principal alkyl esters of spermaceti are therefore C_{28} hexadecyl dodecanoate (cetyl laurate), C_{38} hexadecyl tetradecanoate (cetyl myristate), C_{32} hexadecyl hexadecanoate (cetyl palmitate) and C_{34} hexadecyl octadecanoate (cetyl stearate). The textbook descriptions of spermaceti as being chiefly cetyl palmitate are obviously inadequate. The description of spermaceti as being chiefly cetyl palmitate and cetyl myristate in approximately equal propotions is substantially correct. However, the present experiments have shown that a more accurate description of spermaceti would be a mixture of hexadecyl esters of fatty acids between C_{26} and C_{38} with hexadecyl dodecanoate, hexadecyl tetradecanoate, hexadecyl hexadecanoate and hexadecyl octadecanoate comprising at least 85% of the total esters.

Further experiments showed that the alkyl ester consituents of spermaceti can also be analysed directly by gas chromatography following the injection of a solution of the intact wax. This procedure provides a rapid qualitative and quantitative evaluation of wax samples ideally suited for routine screening.

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J. Pharm. Pharmac., 1968, **20**, 780–789 Received March 28, 1968

The effect of emulsifier concentration on the rheological properties of acacia emulsions

E. SHOTTON AND S. S. DAVIS*

The effect of potassium arabate concentration on the rheological properties of liquid paraffin emulsions has been examined over a range of emulsifier concentrations and volume fractions. The emulsions were of small particle size. Aggregation of the emulsions occurred when the volume fraction (ϕ) was in the region of 0·33–0·43, the exact value of ϕ depending on the emulsifier concentration. The aggregated emulsions demonstrated irreversible shear thinning and hysteresis loops were obtained. The aggregation only occurred during the emulsification process and was due to a macromolecular bridging mechanism. The emulsifier concentration influenced aggregation and thereby influenced the rheological properties of the emulsions by two opposing mechanisms. It decreased the volume fraction at which aggregation first occurred (ϕ_{agg}), through its influence on particle size, but increased ϕ_{agg} through its effect on the degree of contraction of the acacia molecule.

INTERFACIAL films formed by hydrocolloids, such as acacia, were first described by Serrallach & Jones (1931). The build up of these films, their structure and thickness have been investigated by Shotton and his colleagues (Shotton, 1955; Shotton & Wibberley, 1959, 1960, 1961; Wibberley, 1962; Shotton, Wibberley & Vaziri, 1964). They found that the film produced by acacia at the oil-water interface was formed in about 20 sec for solutions of widely different strengths and had elastic properties. It was concluded that the first layer of acacia deposited at the interface was irreversibly adsorbed while subsequent layers were reversibly adsorbed. A film thickness of 0·15 μ was calculated. The stabilization of emulsions by hydrocolloids was dependent mainly on the coherence, rigidity, elasticity and ready adsorption of the interfacial film.

The flow curves of acacia emulsions have been described by Shotton & White (1960, 1963), who found that the emulsion viscosity increased with volume fraction (ϕ), leading to deviations from Newtonian flow but with no evidence of hysteresis. An increased viscosity caused by homogenization was attributed to the thick interfacial film formed by acacia at the oil-water interface. The concentration of acacia appeared to have little effect on relative viscosity but emulsions containing less than 10% w/v acacia were unstable.

Experimental

MATERIALS

Liquid paraffin B.P.; water from an all glass still; potassium arabate prepared from acacia by the method of Shotton (1955).

APPARATUS

A Couette viscometer (Shotton & Davis, 1968a) was used for emulsion systems of low viscosity (<30 cP) and a Ferranti-Shirley viscometer (Van Wazer, Lyons & others, 1963) for those of higher viscosity. Measurements with the Couette viscometer were restricted to Newtonian systems

From the Department of Pharmaceutics, The School of Pharmacy, University of London, 29/39 Brunswick Square, London, W.C.1, England.

* This work formed part of a thesis for the degree of Ph.D. in the University of London.

so that results from the two instruments could be compared directly without complications due to differences in geometry. The maximum shear rate was 120 sec^{-1}. The Ferranti-Shirley equipment was used in conjunction with an automatic flow curve recorder unit designed to provide an standardized shearing procedure. A maximum shear rate of 1692 sec^{-1} and sweep time (upcurve) of 600 sec were chosen. The resultant flow curves were displayed on a Scientific Furnishings Ltd. X-Y autoplotter.

The particle size and particle size distribution of the emulsions were obtained using a model A (Industrial) Coulter Counter.

PROCEDURE

The emulsions were prepared in the manner described by Shotton & Davis (1968a), by dispersing liquid paraffin in solutions of potassium arabate at arabate concentrations 0·75–12·0% w/w aqueous phase. Formulation details are in Table 1. The systems were stored for four days after preparation at 25° to allow for the release of entrapped air and the attainment of interfacial equilibrium (Shotton & White, 1960).

TABLE 1. POTASSIUM ARABATE, LIQUID PARAFFIN SYSTEMS, VISCOSITY AND PARTICLE SIZE RESULTS

Arabate conc. % w/w	ϕ	Relative viscosity		Loop area cm^{2}*	Particle size		Rheological behaviour
		η_{lim}	η_{app}		MVD μ	s.d. σ	
0·75	0·11	1·59	—	—	4·66	2·12	N
	0·22	2·17	—	—	5·04	2·23	N
	0·33	4·20	—	—	3·57	3·76	N
	0·43	7·12	7·30	—	8·35	4·85	P/P
	0·53	9·30	10·4	—	21·5	2·01	P/P H?
	0·63	13·8	18·5	—	28·3	2·13	P/P H?
2·25	0·11	1·76	—	—	3·84	2·26	N
	0·22	2·58	—	—	4·84	2·26	N
	0·33	5·03	—	—	6·31	2·19	N
	0·43	8·33	9·59	30	13·8	1·54	P/P H
	0·53	11·9	15·1	27	14·5	1·62	P/P H
	0·63	18·6	24·4	30	17·0	1·75	P/P H
4·5	0·11	1·52	—	—	4·02	2·38	N
	0·22	2·37	—	—	6·04	2·18	N
	0·33	5·83	—	—	5·89	2·19	N
	0·43	8·84	11·7	31	13·5	1·60	P/P H
	0·53	12·5	18·1	40	12·3	1·63	P/P H
	0·63	20·6	26·9	65	13·6	1·70	P/P H
8·0	0·12	1·48	—	—	3·78	2·28	N
	0·23	2·40	—	—	5·27	2·05	P/P
	0·33	5·31	6·92	—	9·91	2·28	P/P
	0·44	8·60	11·0	54	10·3	1·65	P/P H
	0·54	13·4	19·3	115	13·6	1·62	P/P H
	0·64	20·3	30·3	155	16·8	1·74	P/P H
12·0	0 12	1·67	—	—	4·61	2·24	N
	0 23	2·57	2·58	—	4·88	2·10	P/P
	0 34	5·00	5·99	57	9·46	2·01	P/P H
	0 44	8·71	10·8	132	11·0	1·72	P/P H

N = Newtonian. P/P = Pseudoplastic. H = Hysteresis loop.
* 1 cm^{2} corresponds to 6·5 × 10^{3} dyne cm^{-2} sec^{-1}.

Emulsions containing high concentrations of oil and potassium arabate could not be examined as the high viscosity of such systems presented difficulties in homogenization and the entrapping of air.

Results

MACRO AND MICROSCOPIC APPEARANCE

After four days storage no creaming of the emulsions was observed when the volume fraction of oil was less than 0·3. The serum turbidity of the emulsions that had creamed decreased with increase in ϕ. Examination under the microscope showed that aggregation of the emulsions began when ϕ was in the region of 0·33–0·43. The exact value of ϕ was dependent on emulsifier concentration. The greater the concentration the lower the volume fraction for aggregation (ϕ_{agg}). For volume fractions below 0·3 the emulsion droplets were individually dispersed. The average size of the *individual* droplets decreased with increase in emulsifier concentration. The droplets in those systems containing 0·75% of arabate were larger than those in the other unaggregated systems, but there was no evidence of oil separation.

VISCOSITY

The results of rheological analysis are given in Table 1. Newtonian behaviour was demonstrated by the unaggregated systems of low volume fraction, but non-Newtonian behaviour began in the region of $\phi = 0{\cdot}33$–0·44. Initially this was pseudoplastic in nature but hysteresis loops were obtained as ϕ rose. All the curves passed through the origin and a typical example is shown in Fig. 1. The flow curves were repeated after the sample had been allowed to rest for 10 min but there was no evidence

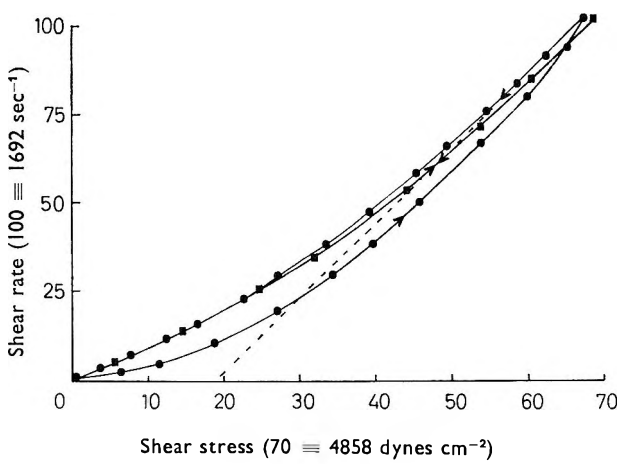

FIG. 1. A typical flow curve for a non-Newtonian arabate system (arabate concentration = 8%, $\phi = 0{\cdot}63$). ● Initial flow curve. ■ Repeat flow curve.

of reformation of thixotropic structure during this time (Fig. 1). The slight movement to the right was due to evaporation, an effect which prevented the emulsions from being rested for longer periods. After shearing, the emulsions were re-examined using the Coulter Counter and a reduction in aggregate size of about 50% was found. The emulsions could be described as being "irreversible shear thinning" in their

rheological properties although it was possible that they had thixotropic structure that reformed slowly.

Limiting viscosities (η_{lim}) and apparent viscosities (η_{app}) for the pseudoplastic systems were calculated in the manner described by Shotton & Davis (1968a). The limiting viscosities of the hysteresis systems were obtained by measuring the gradient of the asymptote to the linear portion of the down curve (Fig. 1). The hysteresis loop areas were measured by planimeter.

The variation of the relative limiting viscosity (η_{rel}^{lim}) with ϕ followed the relation of Richardson (1933) at arabate concentrations 2·25–12·0% (Fig. 2)

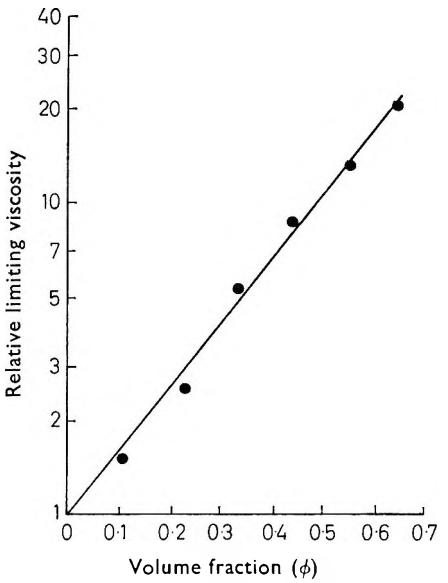

FIG. 2. The variation in relative limiting viscosity with volume fraction for potassium arabate–liquid paraffin emulsions (log plot). Mean results for arabate concentrations 2·25–12·0%.

$$\log \eta_{rel}^{lim} = k\phi$$

where k is a constant, in this case = 2·2. η_{rel}^{lim}, which was calculated from the slope of the linear portion of the down curve and was a measurement of viscosity when structure had been broken down, was independent of arabate concentration for $\phi < 0{\cdot}53$. At higher values of ϕ it increased with arabate concentration up to 8% after which it was again independent. η_{rel}^{app} was calculated from the ratio of shear stress to shear rate at the maximum shear rate, and, as a result, was greatly influenced by the shape of the pseudoplastic up-curve and therefore gave an indication of the amount of aggregation in an emulsion. Its value increased with arabate concentration from $\phi = 0{\cdot}33$ upwards and showed that for a given value of ϕ the greater the arabate concentration, the greater was the aggregation. This was in agreement with the observation above that the exact value of ϕ_{agg} was dependent on arabate concentration.

At and above an arabate concentration of 4·5%, the hysteresis loop area increased almost linearly with volume fraction (Table 1) and the greater the arabate concentration—the greater the area at any value of ϕ. For 2·25% arabate the loop size was independent of ϕ.

The analysis of Coulter Counter results for emulsions has been discussed by Shotton & Davis (1968b). The aggregates formed at high dilution were not broken down during the dilution procedure for counting and could therefore be sized in their original form. Both the aggregated and unaggregated emulsions could be represented by the log-normal distribution of particle size if allowance was made for truncation (Shotton & Davis 1968b). A plot of mean volume diameter (MVD) against volume fraction for arabate concentrations 2·25–12·0% (Fig. 3) shows that aggregation of the emulsions occurred when ϕ was in the region of 0·33–0·43, the exact value of ϕ varying with arabate concentration.

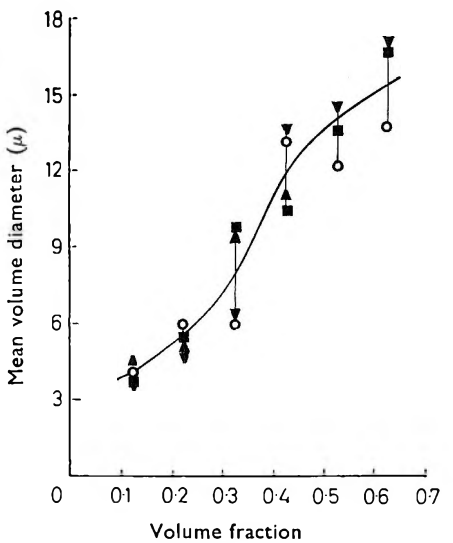

FIG. 3. The change in mean volume diameter of potassium arabate–liquid paraffin emulsions with volume fraction. Arabate concentration % (w/w). —▼— 2·25, —○— 4·5, —■— 8·0, —▲— 12·0.

THE EFFECT OF PARTICLE SIZE ON VISCOSITY

Emulsion homogenization was examined for both the aggregated and the non-aggregated systems (ϕ = 0·53 and 0·22) at arabate concentrations of 1·0, 3·0 and 6·0% w/w. Homogenization of the non-aggregated systems reduced the MVD (Table 2). At 1·0 and 3·0% arabate concentration the viscosity rose to a maximum at a MVD of 13·2 and 13·1 μ respectively, and then fell. 6·0% arabate showed a slight decrease in viscosity upon homogenization (the first homogenization in this case gave a MVD of 10·4 μ). Aggregated emulsions also demonstrated a maximum viscosity, but only after 5–7 passages through the homogenizer. The size of the resultant aggregates decreased with homogenization but the number of

TABLE 2. THE HOMOGENIZATION OF ARABATE EMULSIONS

Non-aggregated systems ($\phi = 0{\cdot}22$)				Aggregated systems ($\phi = 0{\cdot}54$)					
						Viscosity			
Arabate conc. % w/w	Homogn.	Viscosity (cP)	MVD μ	Arabate conc. % w/w	Homogn.	η_{lim} (cP)	η_{app} (cP)	Loop area cm²*	MVD μ
1·0	1	6·43	18·3	1·0	1	16·8	20·8	4	15·1
	2	8·05	13·2		2	22·6	27·2	18	14·4
	3	7·77	12·9		3	23·5	28·6	17	13·8
	4	7·40	11·8		4	23·7	28·7	17	13·0
	5	7·19	9·7		5	24·3	29·0	16·5	12·8
	6	6·41	8·6		6	25·3	30·4	17·5	12·5
	7	6·11	7·6		7	24·1	29·1	14	11·7
3·0	1	8·90	14·4	3·0	1	31·9	35·5	18	13·3
	2	12·8	13·1		2	40·5	48·5	26	12·7
	3	12·0	10·5		3	45·6	48·8	40	11·9
	4	11·8	9·4		4	58·1	63·1	47	10·3
	5	11·1	7·8		5	54·0	60·8	45	9·9
	6	9·8	6·1		6	51·8	57·0	39	9·7
	7	8·8	5·5						
6·0	1	14·2	10·4	6·0	1	52·7	63·6	31·5	12·8
	2	13·7	9·3		2	78·2	99·9	58·5	12·1
	3	13·6	6·3		3	92·3	104·0	63·0	9·9
	4	13·4	5·0		4	86·9	114·0	63·0	8·9
	5	13·4	4·1		5	65·7	87·4	52·5	8·2
	6	13·1	3·5						
	7	13·1	3·2						

* 1 cm² corresponds to $6{\cdot}5 \times 10^3$ dyne cm^{-2} sec^{-1}.

TABLE 3. AGGREGATION OF ARABATE EMULSION WITH TIME $\phi = 0{\cdot}53$, ARABATE CONC. = 3·0% w/w

Time after preparation		1 min	30 min	90 min	1 day	4 days
Particle size	MVD μ	12·66	12·92	12·91	12·92	13·10
Results	s.d. σ	1·56	1·55	1·55	1·55	1·55

TABLE 4. THE STORAGE OF POTASSIUM ARABATE EMULSIONS

Arabate conc. % w/w	ϕ	Particle size results			
		4 days		3 months	
		MVD μ	s.d. σ	MVD μ	s.d. σ
0·75	0·11	4·66	2·12	4·53	1·97
2·25		3·84	2·26	3·52	2·38
4·5		4·02	2·38	3·97	2·40
8·0		3·30	2·37	2·92	2·36
12·0		4·40	2·16	4·40	2·56
0·75	0·43	8·38	4·86	32·8	2·66
2·25		13·8	1·54	15·08	1·62
4·5		13·5	1·60	11·27	1·57
8·0		10·34	1·59	10·88	1·62
12·0		11·88	1·62	17·00	1·79

aggregates was increased. The hysteresis loop areas showed a similar effect to the viscosity results.

THE STORAGE OF EMULSIONS AND ITS EFFECT ON AGGREGATION

The rate of aggregation of a high volume fraction emulsion was studied by sizing samples with the Coulter Counter at different times after preparation (Table 3).

The results show that there was little change in aggregate size in the period 1 min to 4 days. Aggregation must therefore occur during, or immediately after, preparation. At the end of a three month storage period all the emulsions, except those containing 0·75% arabate, at high volume fraction, showed very little change in particle size (Table 4). There is no evidence of aggregation occurring in the originally unaggregated emulsions ($\phi < 0{\cdot}3$). The slight fall in particle size in some cases was probably due to bacterial growth.

Discussion

A comparison of the results obtained in the present work is made with those of White (1961) in Table 5.

TABLE 5. THE RHEOLOGY OF ACACIA EMULSIONS

	Results for present study	White (1961)
(i) Aggregates	Occurred when $\phi > 0{\cdot}33$	Did not occur at any value of ϕ
(ii) Hysteresis loop	Obtained at higher values of ϕ	Pseudoplastic behaviour only
(iii) Emulsion stability	At arabate conc > 2·0%	At arabate concentrations > 10%
(iv) Increase of viscosity with ϕ	For arabate conc. = 8%. $\eta_{rel}^{lim} = 1{\cdot}5$ at $\phi = 0{\cdot}11$, and $\eta_{rel}^{lim} = 11{\cdot}2$ at $\phi = 0{\cdot}5$	For arabate conc. = 15% $\eta_{rel}^{lim} = 1{\cdot}29$ at $\phi = 0{\cdot}1$ and $\eta_{rel}^{lim} = 2{\cdot}99$ at $\phi = 0{\cdot}5$
v) Influence of arabate concentration	Effected value of ϕ at which aggregation occurred and hence value of η_{rel}^{app}	Little or none
(vi) Mean number diameter of droplets	1·0 μ (unaggregated systems)	15·0 μ

The great contrast between the two sets of results may be attributed to aggregation of acacia emulsions when the particle size is small.

In the present work, homogenization of the emulsions resulted in a maximum viscosity. Shotton & White (1963) concluded that the adsorption of a thick interfacial film onto the new droplets formed in a homogenization process would raise the volume fraction of an emulsion and hence the viscosity, and this would explain the increase in viscosity for the non-aggregated systems in the present work. The reduction in particle size may also have promoted the formation of a small number of aggregates which would be broken down irreversibly by further homogenization, thereby causing a fall in viscosity. A similar effect has been reported by Sumner (1954) for the two stage homogenization of milk where the second homogenization produced a decreased viscosity that was probably due to the destruction of aggregates produced in the first homogenization.

The main contribution to the increase of viscosity for the aggregated emulsions would be the creation of an increased number of particles and thus the number of interparticulate "links". After repeated homogenization more aggregates were broken down than new aggregates formed and the viscosity fell.

The nature of these links is important. It is known that emulsions can form aggregates by a number of differing mechanisms and the interparticulate links can be formed either by electrical double layer effects

(Derjaguin, 1940; Verwey & Overbeek, 1948) or by some physical mechanism such as film-film interaction by hydrophobic bonding (Cockbain, 1952, Shotton & Davis, 1968a) or by large molecules bridging two particles (Davies, 1964, Smellie & LaMer, 1958). The nature of the links formed by arabate and the mechanism of aggregation is now discussed.

THE MECHANISM OF AGGREGATION

The aggregates in the arabate emulsions had a number of interesting properties: (i) the aggregates appeared to be broken down irreversibly under shear. (ii) The aggregates were not broken down on dilution, which is in contrast to soap aggregated emulsions (Shotton & Davis, 1968b). (iii) Aggregate formation was dependent on the volume fraction and particle size. (iv) Aggregates were only formed during or immediately after emulsification and non-aggregated systems did not form aggregates even when stored for long periods.

The critical volume fraction [effect (iii)] suggests that the interparticulate distance was important and that in dilute emulsions the particles were too far apart for "links" to be formed in the short period available for aggregation. The distance between particles at varying volume fraction for a given packing distribution may be obtained from the equation

$$\delta = D[(K/\phi)^{\frac{1}{3}} - 1] \quad .. \quad .. \quad .. \quad (1)$$

where δ is the interparticulate distance (μ), D the particle diameter (μ) and K the maximum packing density for the distribution. Thus the smaller the particle size the smaller will be the interparticulate distance for a given volume fraction.

For equisized spheres in rhombohedral array, K has a value of 0·74, whereas for equal spheres in random packing, K is 0·64 (Scott 1960) and for sheared suspensions K is 0·67 (Rutgers, 1962). Emulsion systems, in which the particles are polydisperse, have higher maximum packing density values. Shotton & Davis (1967) found that K has a value close to 0·74 for emulsions in random packing, a value that had been used by Sherman (1960, 1964) in calculating interparticulate distances. For the unaggregated systems in the present work a mean number diameter in the region of 1 μ has been found, so that for an initial aggregation volume fraction of 0·43 and K = 0·74, the interparticulate distance, when aggregation commences, is in the region of 0·20 μ. If for the case of the 15 μ particles reported by White we assume a critical interparticulate distance for aggregation of 0·20 μ, then the critical volume fraction for aggregation is in the region of 0·73, thus explaining the lack of aggregates in White's emulsions.

Davies (1964) considered that the aggregation of emulsions by macromolecules was due to bridge formation between the particles with the forces of interparticulate repulsion balanced by the forces of adsorption at each end of the macromolecule. Hiestand (1964) and Gillespie (1960) have discussed the flocculation of powders by this method. Smellie & LaMer (1958) found that polymer molecules formed a bridge by adsorption of a different portion of the macromolecule on to each particle and

that bridges could only be formed when the surface was partly covered by macromolecules. Polymer adsorption has been treated theoretically by Silberberg (1962) with the polymer thought of as consisting of active centres spaced along the "backbone" of the molecule. Loose ends are able to stick out into the vehicle but at equilibrium, which may be reached slowly by large molecules, these ends are adsorbed. This theory has been verified experimentally by Curme & Natale (1964).

An interesting study was made by Kragh & Langston (1962) on the aggregation of quartz by gelatin. If the particles were deflocculated and kept apart by agitation, the free macromolecule chains were adsorbed onto the deflocculated particle so that bridge formation was no longer possible and the deflocculation was irreversible.

In the present study, the aggregation of potassium arabate emulsions is explained by links which are formed by a molecular bridge mechanism with adsorption of parts of the arabate molecule onto different oil droplets. Wibberley (1963) has shown that the arabate film at the oil-water interface is formed in about 20 sec and from Smellie & LaMer's free surface requirement, bridging is therefore only able to occur in this short period. The interparticulate distance will play an important part in this type of aggregation, as only those particles that are close enough to be bridged by an arabate molecule will be aggregated. When the aggregates are sheared, the adsorbed ends of the molecule will be removed from one or both of the particle surfaces and rearrangement of the molecule (Kragh & Langston, 1962) and the "free surface requirement" (Smellie & LaMer, 1958) will prevent further bridge formation. The emulsions will thus demonstrate irreversible shear thinning. The critical interparticulate distance for aggregation from equation (1) suggests that the length of the linking molecule will be of the order of $0{\cdot}20\,\mu$. This is in agreement with the work of Veis & Eggenberger (1954) and Anderson & Rahman (1967) who have shown that the acacia molecule in solution exists as a stiff coil with a root mean square, end-to-end dimension varying from $0{\cdot}06\,\mu$ at zero charge to $0{\cdot}24\,\mu$ at maximum charge. At the concentrations employed in the present work the coil will be partly unfolded as a result of interparticulate repulsion between ionized carboxyl groups on neighbouring glucuronic acid residues, and an elongated molecule of $0{\cdot}20\,\mu$ is quite probable.

The results have shown that the emulsifier concentration affects the value of ϕ at which aggregation occurs; in general the higher the concentration, the lower the value of ϕ_{agg}. In fact an increase in emulsifier concentration will influence aggregation and thus the rheological properties of an emulsion, by two opposing mechanisms. It will lead to a decrease in particle size, as demonstrated by microscopic examination and by the work of Shotton & Davis (1968b), and thus a decrease in interparticulate distance that will bring about aggregation at lower values of ϕ (eqn 1). On the other hand an increase in emulsifier concentration will lead to a decreased ionization and thus a decreased length of the arabate molecule (Veis & Eggenberger, 1954). A greater volume fraction will therefore be required for initial aggregation.

The effect of concentration on particle size will be the dominant factor as can be seen from rearranging equation (1):

$$\phi_{agg} = 0{\cdot}74/(1 + \delta/D)^3 \quad .. \quad .. \quad .. \quad (2)$$

The interparticulate length, i.e. the length of acacia molecule, has the limits 0·12 and 0·48 μ and a change in molecule size will only be significant when D is small. Only emulsions of small globule size and low arabate concentration will be affected by the length of the arabate molecule. In practice these systems are difficult to prepare and are unstable.

The rapid and secure adsorption of potassium arabate at the oil-water interface has yet to be explained for the arabate molecule has no groups generally recognized as being lipophilic. Kane, LaMer & Linford (1964) have had similar difficulties in explaining the way weakly anionic polymers adsorb onto strongly negative solid surfaces and cause flocculation.

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J. Pharm. Pharmac., 1968, **20**, 790–792 Received March 18, 1968

A note on the solubility of griseofulvin

P. H. ELWORTHY AND F. J. LIPSCOMB

The solubility of griseofulvin in heptane, water and benzene over the temperature range 15–45° is reported together with the thermodynamic properties for solution of the antibiotic in these solvents.

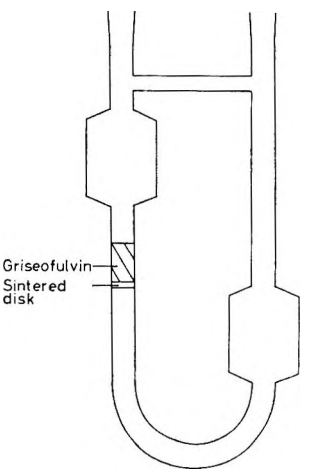
AS part of a study on the rate of solution of griseofulvin, solubilities in various solvents were required. These are reported here together with data on the thermodynamics of solubility.

Experimental

Griseofulvin was recrystallized from ethanol and had m.p. 222°, assay by the method of the British Pharmacopoeia gave a purity of 99·7%. Analar benzene, stored over sodium had n_D^{20},1·5010 (1·5011). Fluka heptane (⩾99 mole %) stored over sodium had n_D^{20}, 1·3880 (1·3876). Literature values of the refractive index are from Timmermans (1965).

Solubility determinations. Preliminary solubility determinations, in which the saturated solution was freed from excess solute on 0·45 μ pore size Millipore filters, were poorly reproducible.

Filtration on a 5/3 sintered glass disk with the minimum of applied pressure gave the best results. A percolation apparatus was devised (Fig. 1) in which the solvent percolated through a plug of griseofulvin supported on a 5/3 sintered glass disk. After the solution had passed through the filter, it was transferred back to the left hand limb of the apparatus and allowed to percolate a second time, a portion being reserved for measurement of extinction. The process was repeated until the solution was saturated, shown by no further increase in extinction. The time for saturation depended on the solvent used, but was in general



From Department of Pharmaceutical Technology, University of Strathclyde, Glasgow, C.1, Scotland.

5–6 days. The percolator was placed on a thermostat, controlled to $\pm 0{\cdot}05°$.

The concentrations of griseofulvin in water and heptane were determined spectrophotometrically by diluting a known weight of the saturated solution with a known amount of ethanol and benzene respectively, and establishing a calibration curve in the ethanol–water and benzene–heptane mixture. In benzene, the solubility was large enough for the E (1%, 1 cm) to be measured directly. Beer's law was obeyed up to saturation. Extinctions were determined using a Uvispek at 296 mμ in water, 287 mμ in heptane, and 291 mμ in benzene.

Results and discussion

The solubility results expressed as molalities (m) are given in Table 1.

TABLE 1. SOLUBILITIES (MOLES/1000 G SOLVENT) OF GRISEOFULVIN IN HEPTANE, WATER AND BENZENE

Solvent	Temperature			
	15°	25°	35°	45°
Heptane ($m \times 10^5$)	0·82	1·49	2·84	5·38
Water ($m \times 10^6$)	1·83	2·45	3·51	6·14
Benzene ($m \times 10^2$)	2·63	3·81	5·14	6·78

In all solvents studied, the solubility increased with temperature. The results for water are in reasonable agreement with those of Bates, Gibaldi & Kanig (1966). The partial molal heats ($\Delta\bar{H}$) and entropies ($\Delta\bar{S}$) of solution are given in Table 2. $\Delta\bar{H}$ was obtained from graphs of log m vs 1/T, and $\Delta\bar{S}$ by dividing $\Delta\bar{H}$ by T. The thermodynamic properties represent the energy change occurring when one mole of griseofulvin is dissolved in an infinite amount of saturated solution.

TABLE 2. THERMODYNAMIC PROPERTIES FOR SOLUTION OF GRISEOFULVIN IN HEPTANE, WATER AND BENZENE

		Heptane	Water	Benzene
20°	$\Delta\bar{H}$	10·9	5·1	5·4
	$\Delta\bar{S}$	37	17	18
30°	$\Delta\bar{H}$	11·6	6·5	5·5
	$\Delta\bar{S}$	38	22	18
40°	$\Delta\bar{H}$	12·4	10·0	5·2
	$\Delta\bar{S}$	40	32	17

$\Delta\bar{H}$ in kcal mole^{-1}, $\Delta\bar{S}$ in cal mole^{-1}deg^{-1}.

In the griseofulvin–heptane system, there is only a small variation of the thermodynamic properties with temperature.

$\Delta\bar{H}$ will include the heat necessary to rupture the bonds in the crystal of griseofulvin, as well as a contribution from heat of dilution, and any interaction with the solvent. In the case of heptane the last two factors

probably make little contribution to $\Delta\bar{H}$. The positive $\Delta\bar{S}$ value indicates the greater disorder of the solute when dissolved over its state in the crystal. For griseofulvin in water, there is likely to be hydrogen bonding between solvent and solute molecules, a process which will tend to affect the overall heat change. $\Delta\bar{H}$ increases with temperature, and as it is known that the structuring of water decreases with temperature rise, the contribution to the heat term from solvent–solute interactions is likely to decrease also. At 40°, $\Delta\bar{H}$ for the griseofulvin–water system is not much less than that of the griseofulvin–heptane system. The increase of $\Delta\bar{S}$ with temperature may also reflect a decrease in the structuring effects of water.

In benzene, the saturation solubility is about one thousand times greater than in the other two solvents, which may introduce effects from heat of dilution in the dissolving process. Benzene is not a structured liquid like water, so strong interactions between solvent and solute are unlikely. There may be evidence for association of griseofulvin molecules in benzene, as both $\Delta\bar{H}$ and $\Delta\bar{S}$ are much lower than in the heptane system. No comment can be made on the change of these quantities with temperature, as the variation is within experimental error.

Acknowledgements. We thank Glaxo Research Ltd., for a research studentship, and for the gift of griseofulvin.

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Similarities in the interatomic distances of some anti-inflammatory agents and inflammagenic amines: a possible insight into their common receptor(s)

SIR,—Histamine and 5-hydroxytryptamine (5-HT) both promote inflammation in small animals, with 5-HT more effective in the rat than in other animals or man (Skidmore & Whitehouse, 1967).

Recently Kier has postulated the preferred conformation of the (17β)-α-ketol side-chain in corticosteroids from molecular orbital calculations of the total energy of the molecule in each of several possible conformations, using the extended Hückel theory (Kier, 1968c). The distances between the hydrogen of the 11β-hydroxy group and each of the two oxo-groups in cortisol (hydrocortisone) in its preferred conformation are rather close to the inter-nitrogen distances in one of the two preferred conformations of histamine (Kier, 1968a) and in 5-HT in its preferred conformation (Kier, 1968b) (see the upper part of Table 1). Kier has therefore postulated that the 11β-hydroxy group hydrogen of cortisol, which is essential for anti-inflammatory activity, could also fit those (inflammagenic) receptor sites which bind the protonated ethylamino-group of histamine or 5-HT, while the 20-oxo- and 3-oxo-groups of cortisol could, respectively, attach to those receptor sites which normally bind the imidazole nitrogen of histamine or the indole nitrogen of 5-HT.

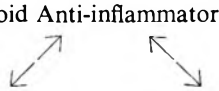
TABLE 1. INTERATOMIC DISTANCES IN CORTISOL, SOME NONSTEROID ANTI-INFLAMMATORY ACIDS (OR THEIR METABOLIC DERIVATIVES), AND INFLAMMAGENIC AMINES

Molecule	Inflammation activity	Distance (Å)
Cortisol	A	20-oxo to 11-oxy-H = 4·8
Histamine	P	Ring N to NH_3^+ = 4·55
Cortisol	A	3-oxo to 11-oxy-H = 6·0
5-HT	P	Ring N to NH_3^+ = 5·84
5-MeO-MIAA*	[A]	Ring N to carboxyl-H = 6·17 or 5·05
3'-Oxophenylbutazone*	A	3'-oxo to ring-ene-3(5)-ol-H = 6·0 or 4·4
N-Arylanthranilic Acids	A	N to carboxyl-H = 4·65
Salicylic Acid	A	Phenolic-O to carboxyl-H = 4·60

* See text
A = antiflammatory; P = pro-inflammatory

This postulate can be extended to include certain nonsteroid anti-inflammatory drugs which, we propose, may be related as in the diagram below. This depicts a pharmacological relation based on possible identity of receptors, the connection lines representing "receptor equivalents".

Nonsteroid Anti-inflammatory Drugs



11β-Hydroxycorticoids ⟷ Inflammatory Amines

Indomethacin, phenylbutazone, the fenamic acids (*N*-arylanthranilates), and salicylates are nonsteroid drugs widely used as alternatives to cortisol (or its more potent derivatives) for treating rheumatoid diseases. They all mimic cortisol in suppressing acute inflammation in experimental animals and the adjuvant-induced arthritis in rats (reviewed by Winter, 1966; Adams & Cobb, 1967; Shen, 1967).

Indomethacin (1,4-chlorobenzoyl-5-methoxy-2-methylindol-3-ylacetic acid) is metabolized in animals, but not apparently in man, with loss of the N_1-chlorobenzoyl moiety (Shen, 1967), possibly through the action of chymotrypsin (Skidmore & Whitehouse, 1967). Molecular orbital calculations indicate that

there are two energetically preferred conformations of the acetic acid side-chain in 5-methoxy-2-methyl-indole acetic acid (MeO-MIAA). The plane of the protonated carboxyl group lies perpendicular to the plane of the ring and two rotamers (preferred conformations) arise because of two energy barriers to rotation of the methylene-carboxyl bond. The distance between the carboxyl hydrogen and the ring-nitrogen in MeO-MIAA is then either 5·05 or 6·17 Å, but this interfunctional group distance cannot be less than 5·0 Å. Therefore, the N_1 and carboxyl-H could not simultaneously bind to the two nitrogen binding sites (4·55 Å apart) of the postulated histamine receptor, but they could simultaneously bind to the two nitrogen-binding sites (5·84 Å apart) of a 5-HT receptor. It is merely coincidental that indomethacin is metabolized to MeO-MIAA in several animal species (rat, rabbit, guinea-pig) but only in the rat, which is uniquely sensitive to 5-HT as an inflammatory mediator, does it have extremely potent activity (at least 80 × phenylbutazone)? In man, indomethacin is not much more potent than phenylbutazone and MeO-MIAA has not been found as a metabolite (Shen, 1967).

Phenylbutazone (1,2-diphenyl-4-n-butyl-pyrazolidine-3,5-dione) is metabolized in man yielding either a phenolic derivative, oxyphenbutazone, or another hydroxy metabolite with a secondary alcoholic group at C-3 in the n-butyl side-chain. This latter compound is claimed to be devoid of anti-inflammatory activity but is chemically labile (see Whitehouse, 1965). The corresponding γ-ketone, another potential metabolite, is an effective antirheumatic agent used, for example, in Czechoslovakia under the name Ketazon. It is a much weaker drug than phenylbutazone *in vitro* in test systems not related to possible amine antagonism (Whitehouse & Leader, 1967). A 3-sulphoxy analogue, sulphinpyrazone (Anturan), is also an effective antirheumatic drug and is more potent than phenylbutazone in suppressing oedema due to formalin or 5-HT in rats (Adam & Cobb, 1967). These experimental observations suggest that a 3′-carbonyl or 3′-sulphoxy group could contribute to drug activity, especially in antagonizing inflammatory amines. A molecular model 3′-oxophenylbutazone, with the 3-oxobutyl side-chain folded in the normal *trans* conformation of paraffin derivatives, shows that the interatomic distances between the hydrogen of a protonated enol group on the pyrazolidine ring and the 3′-oxo group in the benzyl side-chain can be either 4·4 or 6·0 Å, depending on whether the enolic hydrogen is cisoid or transoid with respect to the side-chain. This is remarkable, for it indicates that this one molecule, like cortisol, could possibly interact with both a histamine and a 5-HT receptor. The distances between a ring carbonyl group and the hydrogen atom of a 3-hydroxyl group in the phenylbutazone metabolite from man can be likewise either 4·6 or 5·8 Å.

The salicylate anion is stabilized by an intramolecular hydrogen bond (Baker, 1936) and, if this ion is then protonated, the distance between the phenolic oxygen and the carboxyl hydrogen is 4·6 Å. In the *N*-aryl anthranilic acids mefenamic acid, flufenamic acid, and meclafenamic acid, the distance between the secondary nitrogen and the carboxyl hydrogen is 4·65 Å, assuming a similar hydrogen bond to fix the conformation of the carboxyl group. Thus, both the unionized salicylate and anthranilates might compete with histamine for a receptor site.

These conclusions about possible agonist-antagonist relations presuppose (i) that phenylbutazone and indomethacin are metabolized *in vivo* and that the metabolites may be pharmacologically active *in situ*, even though the same molecular species supplied exogenously are reported to have little or no anti-inflammatory activity (in assays not designed necessarily to detect amine antagonism) probably because they are not similarly distributed or as long-lived *in vivo*

as the parent drugs which engender these metabolites, (ii) that the 11β-hydroxyl of cortisol, the carboxylic hydroxyl group of indole-acetic and other substituted benzoic acids, and the enol group of phenylbutazone are all equivalent to a protonated amino-group, not in any functional sense of reactivity but in a passive binding role, and (iii) it is the unionized form of these nonsteroid drugs that associates with the receptor surface for the inflammagenic amines.

These postulates are not incompatible with previous conclusions that at other receptor sites, not concerned with amine binding, these nonsteroid drugs may be ionized and bound as anions—for these drugs are certainly polyvalent and may influence more than one event in the overall inflammatory process (Whitehouse, 1968). Even though these particular nonsteroids and cortisol may have the requisite intramolecular features for displacing the inflammatory amines from certain receptors, they may not necessarily act as classic anti-5-HT or anti-histamine drugs, that is to say as competitive antagonists; rather, they may serve as false feedback inhibitors to "switch off" amine biogenesis if indeed amine formation is autoregulated by either allosteric or end-product inhibition.

To summarize: It may be significant that both anti-inflammatory steroids, related to cortisol, and nonsteroid anti-inflammatory agents, like fenamates, salicylic acid, and some metabolites or chemical modifications of phenylbutazone and indomethacin, contain oxo- and oxy-groups or oxy and imino-groups so disposed within the molecule that they could also bind to these parts of certain receptors for histamine and 5-HT, that bind the ring nitrogen and ethylamino nitrogen of these two inflammagenic amines.

Biochemistry Division,
Columbus Laboratories
Battelle Memorial Institute,
505 King Avenue
Columbus, Ohio 43201 U.S.A.

LEMONT B. KIER

The College of Pharmacy,
The Ohio State University,
Columbus, Ohio, U.S.A.

MICHAEL W. WHITEHOUSE*

July 26, 1968

* N.S.F. Senior Visiting Foreign Scientist Fellow, The Ohio State University, 1967–1968. Present Address: Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024.

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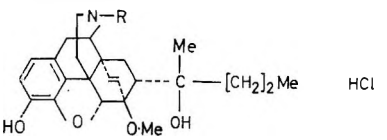
Dissociation of analgesic and respiratory depressant properties in *N*-substituted analogues of etorphine

SIR,—The potent analgesic etorphine (I) shares with all other narcotic analgesics the ability to depress respiratory centres in the brain. This action of the drug, at equinalgesic dose levels, is of greater prominence than that of morphine, both in laboratory species (Blane, Boura & others, 1967) and man (Campbell, McNicol & Lister, personal communication).

Extensive examination of the series of derivatives of tetrahydrothebaine, of which etorphine is a member, has already revealed a number of compounds whose pharmacological characteristics show interesting qualitative and quantitative differences from those of other potent analgesics (Boura & Fitzgerald, 1966; Blane & Boura, 1968). We now find that substitution of the *N*-methyl group in etorphine with allyl, n-propyl, n-pentyl or cyclopropylmethyl groups produces compounds which, whilst retaining morphine-like analgesic properties, are less able to depress respiration. The synthesis of these compounds has already been described (Bentley & Hardy 1967).



I ; R=Me Etorphine
II; R=CH_2—CH=CH_2 R & S 218-M

The hydrochloride of compound (II) [N-allyl-7α-(1-(*R*)-hydroxy-1-methylbutyl)-6, 14 *endo*-ethenotetrahydronororipavine; R & S 218-M] was found to be a potent analgesic after parenteral administration to rats, mice and dogs, using a variety of antinociceptive assays. Thus, given subcutaneously to mice, it was 131 and 100 times more potent than morphine, by the hot plate (Eddy, Touchberry & Lieberman, 1950) or the intraperitoneal phenyl-*p*-benzoquinone (Hendershot & Forsaith, 1959) tests. A similar potency was also found after parenteral administration to rats; the compound caused analgesia at subcutaneous doses which were 52 and 110 times lower than equianalgesic doses of morphine, by the tail pressure (Green & Young, 1951) or inflamed paw (Randall & Selitto, 1957) techniques. A similar action was also caused in dogs, although in this species it appeared less potent. Analgesia could be detected after intramuscular doses approximately 25 times less than those of morphine sulphate (2·5 mg/kg). In each of these test situations the regression lines relating log dose of each drug to intensity of analgesia did not differ significantly from parallelism ($P > 0{\cdot}05$).

Respiratory depression caused by compound II, as indicated by reduction in frequency of respiratory movements, diminished respiratory minute volume and changes in blood tensions of oxygen and carbon dioxide in mice, rats, rabbits and dogs, was significantly less than that caused by either etorphine or morphine at equianalgesic dose levels. Subcutaneously in mice, the doses of the compound and of morphine required to depress respiratory frequency by 50% were 29·5 mg/kg and 14·6 mg/kg respectively, although compound II was found to be more than 100 times more potent than morphine as an analgesic in this species. In rats a subcutaneous dose of morphine sulphate (10 mg/kg) causing analgesia in the radiant heat test (D'Amour & Smith, 1941) elevated venous pCO_2 levels by 49% compared with controls, whereas an equianalgesic dose of compound II (0·1 mg/kg) increased pCO_2 levels by only 19%. Increasing the dose of either drug five

fold resulted in increased pCO_2 levels of 64 and 25% respectively. At both dose levels these differences between the drugs were statistically significant ($P < 0.01$). Significant differences ($P = 0.01$) were also found between the effects of the two drugs on the frequency of respiratory movements, the higher dose level (50 mg/kg) of morphine causing a decrease in respiratory frequency of 22% and an equianalgesic dose of compound II (0·5 mg/kg) a reduction of 11%.

The compound produced less depression of respiration in the newborn than did morphine after administration in equianalgesic doses to pregnant rats at term. By a method previously described (Blane, 1967) the compound was approximately 6 times more potent than morphine as a depressant of neonatal respiration, after being given subcutaneously to the mothers, while it is about 80 times more potent as an analgesic.

In rabbits the mean intravenous doses of etorphine, compound II and morphine required to cause a fall of 20% in arterial pO_2 levels were 0·0008, 4·2 and 4·0 mg/kg respectively. pCO_2 levels were elevated by 20% after mean doses of the three drugs of 0·0008, 3·2 and 6·6 mg/kg respectively. Hence both parameters showed that compound II caused less respiratory depression in the rabbit, relative to its ability to cause analgesia in the other species used, than did either etorphine or morphine. The doses of etorphine, compound II and morphine required to cause equal depression of respiration as measured by altered gas tensions were approximately 1 : 4,600 : 6,600 which was in marked contrast to the relation found between equianalgesic doses of the three drugs in the rat (1 : 27 : 2,400) and in the dog (1 : 40 : 1,000). A further difference was that the maximum intensity of respiratory depression which occurred after very large doses of compound II was significantly less than that produced by large doses of either etorphine or morphine. After large intravenous doses of compound II (4·5 mg/kg) the maximum respiratory depression was a reduction of 27% in respiratory minute volume, a reduction of 20% in blood pO_2 levels, and an increase of 30% in blood pCO_2 levels. Analogous findings using maximal doses of etorphine (4–10 μg/kg) were 60, 40 and 96% respectively, and for morphine (10–20 mg/kg) 58, 38 and 32% respectively. For each parameter the difference between the effect of compound II and that of either of the other analgesics was statistically significant ($P < 0.01$).

Large doses of compound II (5 mg/kg) given intravenously to anaesthetized dogs caused less respiratory depression than the same dose of morphine. Whereas the latter drug reduced the rate and depth of breathing and evoked periodic respiration, after compound II only reduced respiratory rate was seen.

The actions of compound II on the cardiovascular systems of anaesthetized cats and anaesthetized and unanaesthetized dogs resembled those of morphine and also etorphine by inducing bradycardia and lowered arterial blood pressure. Behavioural changes occurring after its parenteral administration to rodents, cats, baboons and dogs were similar to those which occur after either etorphine or morphine. However, in dogs the compound given intramuscularly failed to cause emesis, and in this respect resembles etorphine rather than morphine (Blane, Boura & Fitzgerald, 1967).

The acute toxicity of compound II seems low. The approximate subcutaneous LD50 values in mice and rats were both > 500 mg/kg, whereas for morphine sulphate they were 506 and 170 mg/kg respectively. The morphine antagonist nalorphine, given parenterally, antagonized analgesia in rats and prostration in dogs caused by compound II.

Substitution of the *N*-methyl group by allyl in several other series of narcotic analgesics, for example the morphines, morphinans and benzomorphans, produces compounds which act as competitive antagonists of narcotic analgesics

(de Stevens, 1965). It is therefore of fundamental interest that an analogous change in the molecular structure of etorphine results in a compound which, although less potent, retains powerful analgesic properties whilst showing altered pharmacological characteristics. The evidence obtained from five laboratory species indicating that in compound II there is dissociation of analgesic and respiratory depressant properties, justifies extension of these studies to man.

Department of Pharmacology,
Reckitt and Sons Ltd., Hull,
England.

G. F. BLANE
A. L. A. BOURA
E. C. LEACH

Department of Experimental Pharmacology,
Lederle Laboratories Division,
American Cyanamid Company,
Pearl River,
New York,
U.S.A.

W. D. GRAY
A. C. OSTERBERG

July 18, 1968.

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Pharmacological activity of cannabis according to the sex of the plant

SIR,—It is an old tradition, probably originating in India, that the female plants of *Cannabis sativa* contained more activity than male plants (Walton, 1938). In Brazil some planters used to cut the terminal buds to induce more expanded branches, richer foliage and, consequently, more extractable material. According to a popular belief this *castration* of the pistillate plants must be performed only by men (Doria, 1916). By cultivating marihuana near our laboratories (Valle & Hyppolito, 1964) we were able to gather separately the male and female plants, and to prepare a powder of the leaves and flowering tops, and to store the samples by sex under the same conditions. It was possible, then, to reinvestigate whether there existed a difference of activity between the two kinds of powder.

Leaves and flowering tops were separated from other parts of the plant, dried at room temperature (20–24°), powdered and distributed according to the sex of the plant in dark ampoules, 5 g each, closed under nitrogen and kept in the refrigerator (4°). The crude resin to be assayed was obtained: (*a*) after 4–6 hr Soxhlet extraction with light petroleum (b.p. 50–80°), the extracts being treated with activated charcoal, filtered and evaporated under reduced pressure; (*b*) by extracting the powders at room temperature with the same solvent, the charcoal treatment being omitted before filtration. In both instances the residue

was taken up in acetone and the solution kept overnight at 4° to separate wax constituents. After acetone evaporation the crude resin was dissolved in ethanol and the stock solutions (10 mg/ml) used in the assays. These were made on rabbits using the abolition of the blink reflex (Valle, Souza & Hyppolito, 1966) and by a test based on toxicity to the fish *Lebistes reticulatus* (Valle, Baratella & others, 1967).

The average yield of powder from male and female plants excluding fruits, stem, branches and roots was respectively 11 and 15%. This is not unusual since it is known that at maturity the male plants are more slender and with less foliage than the female ones. Comparative bioassays of the crude resins based on the abolition of the rabbit blink reflex showed that male and female cannabis preparations exhibited practically the same degree of activity, the mean difference not being significant (Table 1). The same was true using the test on the fish.

TABLE 1. COMPARATIVE BIOASSAY OF MALE AND FEMALE CANNABIS CRUDE RESINS BASED ON THE ABOLITION OF THE RABBIT BLINK REFLEX*

Groups	Number of injected animals	Body weight kg (average and limits)	Preparations from	Test solution†	Threshold dosage mg/kg ± s.d.‡
I	6	2·33 (1·5 – 2·9)	male plants	0·5 mg/ml	0·83 ± 0·30
II	6	2·46 (1·9 – 3·2)	female plants	0·5 mg/ml	0·75 ± 0·31

* Repeated intravenous injections (0·2 ml/kg each) at 10 min intervals enough to abolish blink reflex on both eyes.
† Ethanolic stock solution (10 mg/ml) diluted in saline plus polysorbate 80 before using.
‡ Mean difference not significant ($t = 0{\cdot}445 < 2{\cdot}228$, $n = 10$, $p = 0{\cdot}05$).

Although Bouquet (1938) recorded a pharmacological difference in favour of the female cannabis, Houghton & Hamilton (1908), among others, had previously pointed out that staminate and pistillate plants exhibited very similar amounts of activity. We too, by different methods of assay have concluded that cannabis female plants, despite yielding higher amounts of starting drug material, do not exhibit more activity, on a weight basis, than the male plants.

Acknowledgement. We wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support.

Department of Biochemistry and Pharmacology,
Escola Paulista de Medicina,
Caixa postal 20,372,
São Paulo 8, Brazil.

J. R. VALLE
A. J. LAPA
GLAUCE G. BARROS*

June 17, 1968

* With a fellowship from CAPES (Rio de Janeiro).

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The influences of drugs on the uptake of 5-hydroxytryptamine by synaptic vesicles of rabbit brain stem

SIR,—Previous work in our laboratory has shown that reserpine, desipramine and cocaine, when incubated in a medium containing nerve ending particles, 5-hydroxytryptamine (5-HT) and the monoamine oxidase inhibitor, pheniprazine, exerted an inhibitory effect on the uptake of 5-HT by these nerve ending particles (Segawa & Kuruma, 1968). In adrenergic neurons it has been demonstrated that desipramine and cocaine selectively blocked the amine transport through the neuronal membrane while reserpine selectively blocked incorporation into the storage granule (Hillarp & Malmfors, 1964; Carlsson & Waldeck, 1965; Malmfors, 1965). Whether these drugs act at the same sites on 5-HT uptake in 5-HT neurons as with catecholamine uptake in adrenergic neurons is still unknown. We now describe an attempt to see if these drugs have also an inhibitory effect on the uptake of 5-HT by isolated synaptic vesicles.

Male rabbits, of 2·5 kg provided three brain stems (about 7·5 g), which were homogenized in ice-cold 0·32 M sucrose with a Teflon pestle and made up to about 75 ml. The crude mitochondrial P_2-fraction, was prepared by differential centrifugation (Segawa & Kuruma, 1968). The isolation of synaptic vesicles from the disrupted nerve ending particles was made as described by DeRobertis, Rodriguez de Lores Arnaiz & Pellegrino de Iraldi (1962), DeRobertis, Rodriguez de Lores Arnaiz, & others (1963) and Maynert, Levi & De Lorenzo (1964), with some modification. The P_2-fraction was resuspended in 0·32 M sucrose (3 ml/g of original tissue) and diluted to approximately 0·08 M sucrose by the addition of three volumes of ice-cold distilled water. This fraction was homogenized again with a Teflon pestle for about 90 sec at 4° and again centrifuged at 11,500 *g* for 20 min to separate the synaptic ghosts, swollen mitochondria and myelin. The supernatant was then centrifuged at 100,000 *g* for 30 min to sediment the synaptic vesicles fraction. This fraction was separated from the supernatant simply by decantation. Under the electron microscope this fraction appeared to consist mainly of isolated synaptic vesicles contaminated by few membrane fragments.

For 5-HT uptake experiments, the fraction containing synaptic vesicles was suspended in about 40 ml of Krebs solution of pH 7·6. To 4·6 ml of the suspension, 0·2 ml of 5-HT dissolved in phosphate buffer of pH 7·0 and 0·2 ml of test drugs (final concentration of 20 μg/ml) were added. The mixture was then incubated at 37° in air for 60 min. After incubation the mixture was centrifuged at 100,000 *g* for 30 min and the supernantant fluid was decanted. The pellet of synaptic vesicles was directly (or after washing twice with Krebs solution) subjected to 5-HT estimation. 5-HT was extracted and assayed flucrimetrically (Snyder, Axelrod & Zweig, 1965). By electron microscope it was found that most of the synaptic vesicles were unchanged in structure even after 1 hr incubation.

Table 1 gives the results obtained in this experiment. Reserpine and desipr-

TABLE 1. THE EFFECTS OF DRUGS (20 μG/ML) ON THE UPTAKE OF 5-HT AT 37° BY THE FRACTION CONTAINING SYNAPTIC VESICLES FROM THE BRAIN STEM OF THE RABBIT

Drugs	5-HT in medium (μg base/ml)	wash	% change from control
Reserpine	2	0	−26·45*** (4)
Desipramine HCl	2	2	−84·15* (11)
Imipramine HCl	2	2	−78·70** (5)
Cocaine HCl	2	2	−27·80† (5)
	1	2	−34·50† (6)

* $P < 0{\cdot}001$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}02$. † $P > 0{\cdot}1$.

amine which inhibited the uptake of 5-HT by nerve ending particles also showed inhibitory effect on isolated synaptic vesicles. These results imply that if reserpine and desipramine are able to be transported through neuronal membrane they are capable of blocking the intracellular 5-HT concentrating mechanism located at synaptic vesicles level. On the other hand cocaine showed no significant effect on 5-HT uptake by synaptic vesicles even when the concentration of 5-HT in the medium was decreased to 1 μg/ml. This result together with the previous observation that cocaine caused an inhibition of the uptake of 5-HT by nerve ending particles (Segawa & Kuruma, 1968) indicates that cocaine selectively blocks the neuronal membrane pump in the same way as it does with catecholamine uptake. Imipramine was reported to be capable of blocking the 5-HT concentrating mechanism located at the level of cell membrane (Fuxe & Ungerstedt, 1968). Our result showed that imipramine significantly blocked 5-HT uptake by synaptic vesicles under the experimental condition described above.

Department of Pharmacology,
Faculty of Pharmaceutical Sciences,
Kyoto University,
Kyoto, Japan.

TOMIO SEGAWA
ISAMI KURUMA
KATSUYA TAKATSUKA
HIROSHI TAKAGI

July 23, 1968

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Development of antihistamine and anti-allergic activity after prolonged administration of a plant saponin from *Clerodendron serratum*

SIR,—It was of interest that both the alcoholic extract and the saponin isolated from the root bark of an indigenous plant, *Clerodendron serratum*, which has been used for the treatment of bronchial asthma, caused release of histamine from lung tissue (Gupta & Gupta, 1967). Prolonged administration of the saponin in 20 mg/kg doses caused significant depletion of the amine from the lungs of rats treated with the drug (Gupta, Mahesh Rai & Gupta, 1967). The saponin fraction like other histamine releasers was not found to manifest any antihistamine activity or to give protection against anaphylactic shock in sensitized guinea-pigs exposed to egg albumin (antigen) micro-aerosols (Mongar & Schild, 1952). However, the continued daily administration of the drug, 20 mg/kg (1/15 of the LD50 dose 307·7 mg/kg), intramuscularly for 20 days to sensitized guinea-pigs was found to gradually develop protection against anaphylaxis. This became evident from the significant ($P < 0{\cdot}05$) delay in onset of dyspnoea in treated animals exposed to 1·0% egg albumin micro-aerosol as compared to the controls. At the end of 20 days, the treated animals on continued exposure to the micro-aerosol for 10 min, did not manifest air hunger or asphyxial convulsion as was observed in untreated controls before collapse. The results are summarized in Table 1.

TABLE 1. SHOWING THE EFFECT OF PROLONGED ADMINISTRATION OF CLERODENDRON SAPONIN AGAINST ANAPHYLACTIC MICRO-AEROSOLS IN GUINEA-PIGS

	Average time (sec) for onset of dyspnoea			Collapse (preconvulsive time, sec)	Protection %
	Before treatment	1 hr after treatment	10 days after treatment	20 days after treatment	
Control (3)	130 ±28	106 ±22	168 ±5	230 ±36	Nil
Saponin—*C. serratum* (3)	133 ±25	117 ±21	366 ±73	>600	100

The lungs from animals killed were extracted with concentrated hydrochloric acid for 2 hr (Francis, Melville & Douglas, 1963). The filtrate, after extraction successively with light petroleum, ethyl acetate, carbon tetrachloride or ethyl ether, gave an oily substance which on dissolving in equal parts of saline and propylene glycol was tested for antihistamine and anti-allergic activity on isolated strips of guinea-pig ileum.

The constrictor responses to the standard doses of histamine of the isolated strip of ileum suspended in a 10 ml Tyrode bath were found to remain inhibited to below 50% for an average period of 16·0 ± 8·1 min after addition of 2 mg of the lung extract obtained from treated animals, while this inhibition was of much shorter duration (6·3 ± 2·6 min) after addition of the equivalent doses of the extracts from control animals. The lung extracts from treated animals were significantly more effective compared with controls in inhibiting the constrictor responses of slow reacting substance obtained from perfusion of the sensitized lung with egg albumin. Development of the antihistamine and anti-allergic activity observed in the treated animals may be as a result of adaptation to chemical stress induced by release of histamine by the drug, in view of the fact that development of a "Resistin" like substance has been reported by Karady, Kovacs & others (1957). These observations seem to throw some light on explaining the beneficial effects of the plant saponin in bronchial asthma and might also suggest the use of non-toxic histamine releasers in the treatment of allergies, the possibility of which was indicated earlier by Gaddum (1948).

Department of Pharmacology,
Gandhi Medical College,
Bhopal, India.

S. S. GUPTA

July 6, 1968

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The survival of heat resistant spores in aqueous injections after an official sterilization treatment

SIR,—Differing concentrations of spores of *Bacillus stearothermophilus* were prepared and counted as described by Cook & Gilbert (1965). Suspensions of spores in 1 ml volumes of water for injection B.P. and sodium chloride injection B.P. were heated in ampoules at 115° in an oil bath (Gilbert 1966) for between 10–120 min. The ampoules were then cooled in water at 4°, opened, and the contents transferred aseptically to tubes containing 10 ml volumes of tryptone (Oxoid) 1%, dextrose 0·5% and soluble starch 0·1% in water. Soluble starch was included in the medium to aid the recovery of surviving spores and the pH adjusted to 7·3 as recommended by Cook & Gilbert (1968). The usual indicator bromo-cresol purple, included in media to detect flat-sour organisms such as *B. stearothermophilus*, was omitted as recommended by Cook & Brown (1960). The tubes were incubated for 3 days at 50°.

TABLE 1. HEAT RESISTANCE OF *B. stearothermophilus* SPORES AT 115° IN AQUEOUS INJECTIONS

Heating medium 1 ml volumes	Concentration of spores in heating medium*	Tubes of recovery medium yielding growth after heating ampoules				
		Heating time at 115° (min)				
		10	30	60	90	120
Water for injection B.P.	10^6	—	10/10	10/10	7/10	0/10
	10^4	—	10/10	7/10	0/10	0/10
	10^2	10/10	3/10	0/10	0/10	—
Sodium chloride injection B.P.	10^4	—	10/10	2/10	0/10	0/10

* Calculated from plate counts of unheated spores.

Table 1 shows that heating at 115° for 30 min was insufficient for sterilization of water for injection or sodium chloride injection, and the time for sterilization increased with increase in spore concentration in the ampoules. The influence of spore load has also been stressed by Kelsey (1961) using spore strips impregnated with *B. stearothermophilus* spores heated in steam at 121°. *B. stearothermophilus* is a non-pathogen with a very wide growth range of about 37°–70° and is normally associated with thermophilic spoilage of low-acid canned foods.

We do not propose that the B.P. official sterilizing time: temperature relation be altered or that tests for sterility include incubation at 50°, but it should be recorded that low concentrations of spores can survive the B.P. sterilization process in aqueous injections. Spores of important pathogenic organisms e.g. *Clostridium tetani*, *Clostridium welchii*, *Bacillus anthracis*, are readily killed by moist heat at 115°.

School of Pharmacy,
University of London,
Brunswick Square, London, W.C.1.,
England.
July 5, 1968

A. M. COOK*
R. J. GILBERT†

* Present address: Welsh School of Pharmacy, University of Wales Institute of Science and Technology, Cathays Park, Cardiff, Wales.
† Present address: Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue London, N.W.9, England.

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The effect of chlordiazepoxide and fluphenazine on critical flicker frequency

SIR,—The critical flicker frequency (CFF), which is the rate at which a light of increasing intermittency first appears to be flickering, has for many years been used as a sensitive test of central nervous system activity (Landis, 1954; Turner, 1968). CFF not only varies with age and in neurological disorders (Misiak, 1967) but is affected by centrally acting drugs. Earlier studies had used large doses of oral agents, but it has now been shown (Turner, 1968) that single therapeutic doses of several compounds including phenmetrazine, amphetamine, chlorpromazine and amylobarbitone may alter the CFF, and so it seems reasonable to regard measurement of CFF as a potential screening test for centrally acting drugs.

We have now compared chlordiazepoxide and fluphenazine hydrochloride which were shown recently (Rickels, Raab & others, 1968) to be effective in the management of anxiety states. Chlordiazepoxide, 10 and 20 mg, in two divided doses, 4 hr apart, and 2 mg of fluphenazine hydrochloride in a single dose were compared with placebo in a double blind trial. A latin square design of administration was used in eight young volunteers of either sex. CFF, both ascending and descending, was measured before, and at 2 hrly intervals for 8 hr after ingestion. The apparatus and technique were described by Turner (1968).

The results were submitted to an analysis of variance which revealed no significant difference between the two drugs and placebo. However, as has been consistently found with this method, a significant ($P < 0.001$) fall of CFF was observed over the day (Turner, Sneddon & Smart, 1967).

These results are in accordance with those of Idestrom & Cadenius (1963) who found that while a single dose of 20 mg chlordiazepoxide did not significantly reduce CFF, a 40 mg dose did so. Austen, Gilmartin & Turner (1968) have measured the action of chlordiazepoxide (10 mg) on visuomotor co-ordination, visual field, extraocular muscle balance and colour matching ability and found no significant change. Although fluphenazine hydrochloride, 1 mg, has been shown not to alter CFF (Turner, 1966) it is now recommended in doses of 2 mg daily in the management of anxiety states and therefore requires re-evaluation.

Clinical Pharmacology Division,
Medical Professorial Unit,
St. Bartholomew's Hospital,
London, E.C.1.

NORMA A. LIND
PAUL TURNER

July 26, 1968

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Development of tolerance to the behavioural depressant effects of α-methyltyrosine

SIR,—α-Methyltyrosine produces marked behavioural depression in animals. It disrupts conditioned avoidance behaviour in rats, guinea-pigs and cats (Hanson, 1965; Moore, 1966). It also disrupts rotarod performance and spontaneous locomotor activity (Rech, Borys & Moore, 1966), operant behaviour (Schoenfeld & Seiden, 1967) and self-stimulation (Poschel & Ninteman, 1966) in rats. The behavioural depressant effects of α-methyltyrosine appear to be associated with the ability of this compound to deplete the brain of catecholamines (Moore & Rech, 1967; Rech, Carr & Moore, 1968).

Despite the pronounced behavioural depression observed after acute dosage of α-methyltyrosine in animals, minimal central depressant effects were observed after the administration of the drug to man (Charalampous & Brown, 1967; Gershon, Hekimian & others, 1967; Engelman, Horwitz & others, 1968). This lack of central depression after large doses of the drug is puzzling in light of the animal findings. I wondered if the acute findings in animals were maintained after chronic dosage.

Male albino mice (Spartan Farms), 25–30 g were maintained (8 per cage) on a ground diet (Wayne Lab-blox) containing 0, 0·3 or 1% L-α-methyltyrosine. At various times after the feeding program was initiated, spontaneous locomotor activity was measured in circular actophotometer cages (Woodward Research Corporation). Two mice were placed in each cage. After a 10 min period of acclimatization, motor activity was recorded for 10 min. The mice were then decapitated and blood collected from the trunk into beakers containing heparin. Four brains were pooled and analysed for noradrenaline and dopamine (Moore & Rech, 1967). Four plasma samples were pooled and analysed for α-methyltyrosine (Carr & Moore, 1968).

Table 1 summarizes the effects of a 24 hr diet of 0·3 and 1·0% α-methyltyrosine. The results depicted here essentially confirm those reported by Johnson, Kim & others (1967). That is, when administered in the diet, the drug produced a dose-related reduction of spontaneous locomotor activity. This behavioural effect was mirrored by a reduction in the brain contents of noradrenaline and dopamine. The 1% diet, but not the 0·3% diet, of α-methyltyrosine markedly reduced both food intake and body weight. Accordingly, the 0·3% diet was used in all subsequent experiments.

Fig. 1 shows the effects of a chronic diet of α-methyltyrosine on spontaneous locomotor activity, plasma α-methyltyrosine and brain catecholamine levels. The values depicted for day 1 are similar to those presented for the 0·3% diet

TABLE 1. EFFECTS OF A 24 HR DIET OF α-METHYLTYROSINE IN MICE

	N	Control	α-Methyltyrosine 0·3%	α-Methyltyrosine 1·0%
Motor activity (counts/10 min)	16	504 ± 18	212 ± 16*	53 ± 9*
Brain noradrenaline (μg/g)	8	0·35 ± 0·01	0·20 ± 0·01*	0·13 ± 0·01*
Brain dopamine (μg/g)	8	0·71 ± 0·05	0·42 ± 0·04*	0·22 ± 0·04*
% change in body wt	4	+1·5	−0·3	−12·1
Food intake (g/g body wt)	4	0·178 ± 0·020	0·177 ± 0·018	0·079 ± 0·006*
α-Methyltyrosine intake (mg/kg)	4	—	530	785
Plasma α-methyltyrosine (μg/ml)	8	—	6·6 ± 0·4	52·8 ± 8·9

Figures represent the mean ± 1 standard error as determined from 4 cages of 8 animals on each diet. N, the number of determinations.
* Significantly different from control diet at $P < 0.01$.

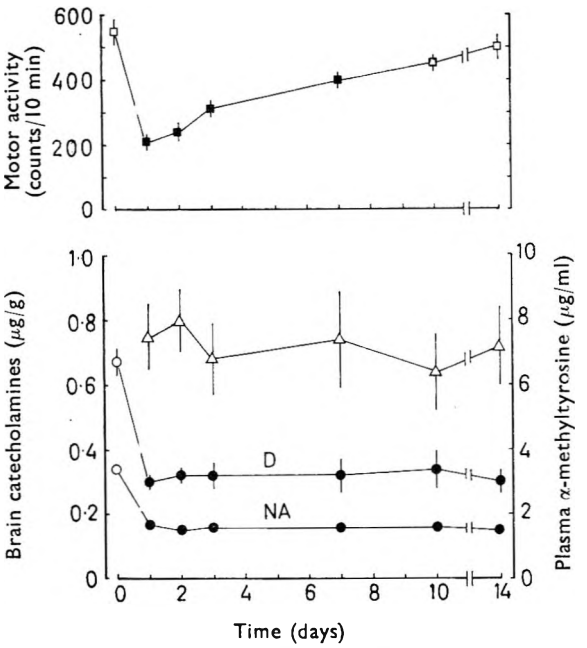

FIG. 1. Effects of a chronic diet of 0·3% α-methyltyrosine on spontaneous locomotor activity, plasma levels of α-methyltyrosine and brain levels of catecholamines. Plasma α-methyltyrosine (△) and brain catecholamines (○) represent the mean of 8 determinations and motor activity (□) represents the mean of 16 determinations; the vertical lines projected upon each point represents one standard error of that mean. Solid points represent those values that are significantly different from control (zero time) at $P < 0{\cdot}01$.

in Table 1. Continued administration of the drug for up to 7 days resulted in significantly lower levels of motor activity. However, this depressant effect diminished with time so that by 10 days and thereafter there was no significant reduction of motor activity. Throughout the two week period there was no alteration in the plasma content of α-methyltyrosine and brain catecholamine levels remained low.

It would seem important to determine the mechanism of tolerance development to the behavioural effect of α-methyltyrosine. Its metabolites (α-methyldopamine or α-methylnoradrenaline) may slowly accumulate in the brain and as "false transmitters" gradually assume the functions of the depleted noradrenaline and dopamine. An alternative explanation would be that during the development of tolerance "central adrenergic receptors" develop an increased sensitivity to noradrenaline. However, regardless of the mechanism, the development of tolerance to the depressant effects of the drug may account for the lack of marked sedative and antipsychotic effects observed when it is administered chronically to man.

Acknowledgements. This work was supported by USPHS Grant AM 11083. L-α-Methyltyrosine was kindly supplied by Dr. C. A. Stone, Merck Institute for Therapeutic Research, West Point, Pennsylvania, U.S.A. The technical asistance of Mrs. M. Gramatins and Mrs. D. Simpson is gratefully acknowledged.

Department of Pharmacology,
Michigan State University,
East Lansing, Michigan 48823, U.S.A.

KENNETH E. MOORE

July 5, 1968

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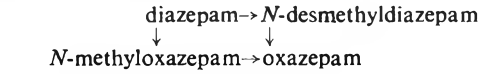
Metabolism of diazepam in isolated perfused liver of rat and mouse

SIR,—It was reported (Schwartz, Koechlin, & others, 1965; Schwartz, Bommer & and Vane, 1967) that in rats, dogs and man the major metabolic pathways of ^{3}H-labelled diazepam involved *N*-demethylation and C-3 hydroxylation, according to the following scheme:



We now confirm the formation of these metabolites by using the isolated perfused liver of rats and mice.

Male Sprague-Dawley rats (200 g) or male Swiss mice (21–24 g) were used as donors of blood and livers. The animals were kept on a standard diet (ALAL 56) and had food and water *ad libitum*. Diazepam or its metabolites were added to the perfusion fluid in concentrations of 50 μg/ml. Details of the technique for the perfusion of the isolated livers of rats and mice have been described elsewhere (Kvetina & Guaitani, 1968). At selected intervals, 2 ml amounts of the perfusion fluid were withdrawn from the circulation and extracted twice with 10 ml of ether. The combined ether extracts, of diazepam and its metabolites were evaporated to dryness.

The residue was dissolved in hexane and quantitatively transferred into a thin-layer plate of Silica Gel G. Pure standards of diazepam and its three metabolites were run alongside the sample extracts for identification of the compounds.

The plates were developed to 15 cm above the origin in glass tanks using the solvent system chloroform acetone, 90 : 10. After development, the plates were allowed to dry completely and then viewed under ultraviolet light (245–350 mμ) to identify the compounds on the plate.

The metabolites formed during the liver perfusion were further identified and verified by two-dimensional chromatography in at least three solvent system pairs (chloroform–heptane–ethanol (10 : 10 : 1), heptane–chloroform–acetic acid–ethanol (5 : 5 : 1 : 0·3), chloroform–acetone (90 : 10)).

The metabolite identification was reinforced by the colour reactions developed

when the plates were sprayed with the Dragendorff reagent modified by Munier & Macheboeuf (1951).

Comparison of the migration of the metabolites extracted from the liver perfusion fluid containing 50 μg/ml of diazepam with known standards indicated that there are no qualitative differences in the liver metabolism of diazepam between rats and mice.

The identified metabolites were: *N*-desmethyldiazepam, *N*-methyloxazepam and oxazepam.

Samples of the perfusion fluid at different times, showed that, at 5 min, only small quantities of *N*-desmethyldiazepam were present. By 15 min in the rat or by 30 min in the mouse, in addition to an increased amount of the above metabolite, *N*-methyloxazepam could also be identified. Oxazepam appeared at 30 min in the rat and at 90 min in the mouse liver perfusion fluid.

Studies on the metabolism of diazepam metabolites using the same technique of the perfused liver showed that the perfusion with *N*-desmethyldiazepam led to the formation of oxazepam both in rat and in mouse. The perfusion with *N*-methyloxazepam resulted in both species in the formation of oxazepam. In addition to oxazepam an unidentified metabolite was present only in the perfusion fluid coming from the liver of the mouse. The perfusion with oxazepam showed that there was no further formation of other metabolites. These results are summarized in Table 1.

TABLE 1. THE METABOLISM OF DIAZEPAM AND ITS METABOLITES IN ISOLATED PERFUSED LIVER OF RATS AND MICE

Compound added	Rf in chloroform–acetone (90:10)	Metabolites identified after 90 min
Diazepam	0·79	NDD, NMO, OX
N-Desmethyldiazepam (NDD)	0·41	OX
N-Methyloxazepam (NMO)	0·63	OX, unidentified*
Oxazepam (OX)	0·17	—

* Only for mouse liver.

Acknowledgement. The authors are grateful to Mr. C. Reschiotto for his skilful technical assistance.

This study was supported by the contract DHEW/PHS/NIH,PH 43–67–83 of the National Institutes of Health, Bethesda, Md., U.S.A.

Istituto di Richerche Farmacologiche
'Mario Negri'
Via Eritrea, 62, 20157 Milan,
Italy

J. KVETINA*
F. MARCUCCI
R. FANELLI

August 1, 1968

* "Visiting Scientist" from Department of Pharmacology, Medical Faculty, Charles University, Hradec Kralove, Czechoslovakia.

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Imipramine affects dopamine uptake by rat brain

SIR,—We wish to present evidence that imipramine affects the uptake of dopamine by brain aminergic neurons. Our evidence is indirect but is consistent with the evidence that the central action of the tricyclic antidepressants is related to the blockade of a membrane concentrating or uptake mechanism as shown for peripheral nerves (Titus, Matussek & others, 1966). In the central nervous system, antidepressants alter the uptake of intraventricularly injected radioactive noradrenaline (Schanberg, Schildkraut & Kopin, 1967; Glowinski & Axelrod, 1964), to decrease the histochemical fluorescence of intraperitoneally injected precursors of noradrenaline and 5-hydroxytryptamine (5-HT), and to reduce the amount of chemically determined amines (Carlsson, Fuxe & Ungerstedt, 1968; Carlsson, Fuxe & others, 1966). We have shown elsewhere that, after intraventricular injection, less 5-HT is taken up by the brain when it is combined with imipramine than when it is given alone (Pscheidt & Himwich, 1968).

Rats were injected intraventricularly (Noble, Wurtman & Axelrod, 1967) with a constant injection volume of 10 μl. At appropriate times the animals were decapitated and the brains rapidly removed, rinsed with saline, frozen on solid carbon dioxide and subsequently analysed for their content of dopamine. The brains were homogenized and extracted (Mead & Finger, 1961) and the final extract treated with iodine (McGeer & McGeer, 1961) and the apparent dopamine content determined fluorometrically. The dosage and injection schedule is in Table 1.

TABLE 1. DOPAMINE CONTENT OF MOUSE BRAIN (μG/G) AFTER TREATMENT WITH TRANYLCYPROMINE, DOPAMINE OR IMIPRAMINE SEPARATELY OR IN COMBINATION

Dose rate and time given before death	Number of animals	Dopamine content of brain, μg/g ± standard deviation
None	6	1·7 ± 0·3
Dopamine (20 μg, i.vent., 30 min)	12	2·4 ± 0·9
Imipramine (500 μg, i.vent., 30 min)	4	1·6 ± 0·5
Tranylcypromine (5 mg/kg, i.p., 0 min)	3	2·4 ± 0·7
Tranylcypromine (5 mg/kg, i.p., 0 min) and imipramine (500 μg, i.vent., 30 min)	3	2·3 ± 0·2
Tranylcypromine (5 mg/kg, i.p., 0 min) and imipramine (500 μg, i.vent., 30 min) and dopamine (20 μg, i.vent., 30 min)	6	3·5 ± 1·1
Tranylcypromine (5 mg/kg, i.p., 0 min) and dopamine (20 μg, i.vent., 30 min)	12	9·8 ± 1·1
Dopamine (20 μg, i.vent., 7½ min)	4	8·7 ± 4·5
Dopamine (20 μg, i.vent., 15 min)	5	5·0 ± 1·9
Dopamine (20 μg, i.vent., 60 min)	6	1·7 ± 0·7

The pertinent findings are the lack of change in brain dopamine with all drug combinations except those of tranylcypromine-dopamine and tranylcypromine-imipramine-dopamine. The former combination elevated brain dopamine content five times above control levels whereas inclusion of imipramine in the intraventricular injection reduced this elevation to only a two-fold increase. We interpret this result as wholly consistent with the view that tricyclic antidepressants are capable of blocking the uptake of dopamine at central aminergic neurons.

Thudichum Psychiatric Research Laboratory,
Galesburg State Research Hospital,
Galesburg, Illinois 61401, U.S.A.

GORDON R. PSCHEIDT
HAROLD E. HIMWICH

July 3, 1968

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Microscopical appearance of some oil-in-water emulsions

SIR,—Barry (1968) has demonstrated the presence of crystalline material in polyhedral particles occurring in emulsions. We find the preparative technique to influence the microscopical appearance of some similar emulsions. Solutions of cetostearyl alcohol (7 g) in liquid paraffin (50 g), and cetomacrogol 1000 or cetrimide (0·5 g) in water (42·5 g) were mixed at 60°. Separate batches of the crude emulsion were (*a*) stirred by hand, (*b*) passed through an automatic pipetting syringe or (*c*) passed through a Q.P. hand homogenizer four times. Samples of each product were examined under a phase contrast microscope and where necessary they were diluted with distilled water.

The relatively gentle shear action of stirring produced emulsions in which we, too, observed globules containing crystalline material.

The syringe technique resulted in a greater degree of globule shearing. Crystals were not visible within the globules but what appeared to be filamentous structures could be seen, either enveloping the globules or dispersed in the aqueous phase (Fig. 1A). These structures were present in the freshly prepared emulsion and did not disappear on ageing or show up under polarized light. They melted when the samples were heated to about 60° on a Leitz hot stage. On cooling, acicular crystals could be seen inside some globules, and filaments were again evident, this time radially orientated at the oil–water interface (Fig. 1B). The orientation effect might have been due to the coverslip but it seems likely that, in general, the interfacial film may act as a template for filament production.

The greatly reduced globule size of the highly sheared homogenized emulsions limited the detail visible by optical microscopy. Aggregates of globules, as noted by Axon (1957) and Riegelman (1962) were apparent in diluted samples. Fig. 1C shows an undiluted emulsion which has been heated to about 60°.

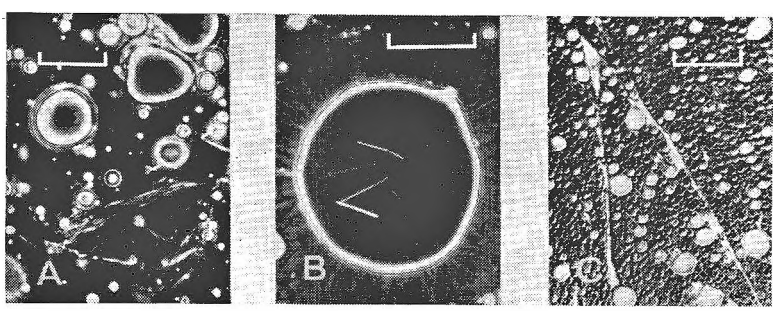

FIG. 1. Photomicrographs of: (A) Emulsion prepared by syringe technique—diluted before examination. One division = 10 μ. (B) As (A) but heated and cooled. One division = 20 μ. (C) Undiluted homogenized emulsion after heating and cooling. One division = 30 μ.

Some coalescence of the globules has occurred and long filaments developed which solidified on cooling.

These experiments were repeated with emulsions containing less than 1·0% w/w cetostearyl alcohol. In no case were we able to demonstrate the presence of filamentous structures.

These observations provide some evidence of migration of cetostearyl alcohol and indicate that its ultimate location is dependent on the previous history of the emulsion. In the aqueous phase the fatty alcohol is probably combined with the surfactant in a liquid crystalline state as described by Barry & Shotton (1967) for a similar system. The filamentous structures shown here are not necessarily of precisely the same form as those of the gel to which we earlier attributed the rheological properties of our emulsions (Talman, Davies & Rowan, 1967; 1968), although they are likely to be of the same constitution. The presence of such a gel is indicated by the aggregates of globules which appear to be immobilized within a matrix and are difficult to disperse. The fine structure of the gel has so far evaded detection, undoubtedly due to the limits of resolution of the optical microscope.

Department of Pharmaceutics,
School of Pharmacy,
Brighton College of Technology,
Brighton, BN2 4GJ, England.

F. A. J. TALMAN
E. M. ROWAN (Miss)

July 5, 1968

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Supersensitivity to tyramine not due to monoamine oxidase inhibition

SIR,—Our observation that chlorobethanidine [1-(*o*-chlorobenzyl)-2,3-dimethylguanidine; BW 392C60], a bretylium-like agent greatly enhanced the responses of the rat isolated vas deferens to tyramine, prompted us to investigate the mechanism involved. Chlorobethanidine differs from bretylium in showing an inhibitory action on monoamine oxidase (Gessa, Cuenca & Costa, 1963). Since most monoamine oxidase inhibitors have been shown to potentiate tyramine responses in a number of preparations (Furchgott, Weinstein & others, 1955; Balzer & Holtz, 1956; Corne & Graham, 1957; Goldberg & Sjoerdsma, 1959; Spano, 1966; Laporte, Jané & Valdecasas, 1968) and it has been postulated that this potentiation is related to monoamine oxidase inhibition (Goldberg & Sjoerdsma, 1959), it could be assumed that the potentiation observed by us could be explained on the basis of its enzyme inhibition. However, taking into account the fact that the doses of chlorobethanidine which determined tyramine enhancement in our experimental conditions were devoid of monoamine oxidase inhibitory activity in the rat heart (Gessa, Cuenca & Costa, 1963), the possibility was entertained that these doses did not inhibit the monoamine oxidase activity of the rat vas deferens. To demonstrate our working hypothesis, the influence of chlorobethanidine on the responses of the rat isolated vas deferens to tyramine or rat vas deferens monoamine oxidase activity, or both, was investigated.

Rats were injected intraperitoneally with different doses of the drug and killed 6 hr later and both vasa were removed. Cumulative dose response curves for

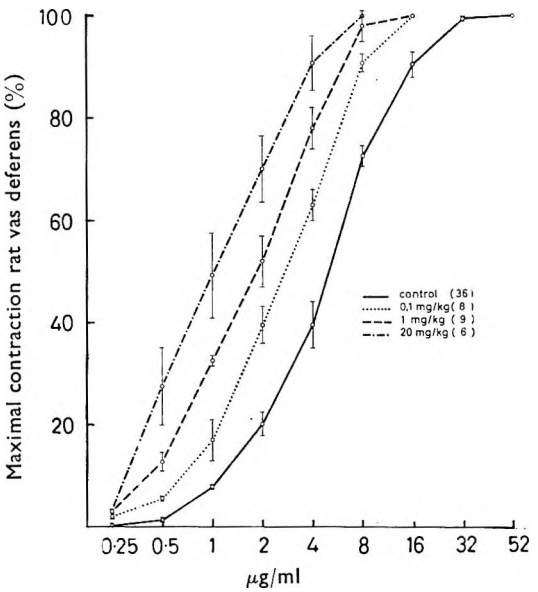

FIG. 1. Modification of the cumulative log concentration-response curves of tyramine by different doses of chlorobethanidine. Rat isolated vas deferens. The mean values and their standard errors are shown. The drug was given 6 hr before the animals were killed. In brackets, the number of experiments.

tyramine were done in controls (36 experiments) and treated animals (23 experiments). Vas deferens monoamine oxidase activity was also measured in some treated animals (Bogdanski, Weissbach & Udenfriend, 1957).

Chlorobethanidine (0·1 mg/kg) greatly enhanced the responses of the rat isolated vas deferens to tyramine (Fig. 1) with the dose response curve clearly shifted to the left. Higher doses (1 and 20 mg/kg) produced a more pronounced supersensitivity (Fig. 1). Vas deferens monoamine oxidase activity in the animals treated 6 hr before with the lower effective doses of the drug (0·1 and 1 mg/kg) showed no difference from that of controls. The 20 mg/kg dose produced 38% inhibition (mean of 3 exp.).

It can then be concluded that the potentiation of the tyramine responses elicited by chlorobethanidine is not related to monoamine oxidase inhibition.

Departamento de Farmacología,
Facultad de Medicina,
Universidad de Barcelona,
Spain.

E. CUENCA
J. FORN
F. G. VALDECASAS

July 28, 1968

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Surface tension effects of β-adrenergic blocking drugs

SIR,—Some β-adrenergic blocking drugs have significant local anaesthetic or membrane effects, while others are devoid of this activity (Ariëns, 1967; Levy, 1968). This property appears to be unrelated to β-adrenergic blocking potency, although some parallelism between local anaesthetic actions and lipoid solubility was observed in a limited series of compounds (Levy, 1968).

In a search for mechanisms underlying the local anaesthetic effect of several of these compounds, their ability to lower surface tension of an aqueous solution was examined because a parallelism between the ability to lower surface tension of an aqueous solution and the local anaesthetic actions of a homologous drug series has been shown (Skou, 1954; Watson, 1960).

I now report an attempt to relate the surface tension lowering effect of five β-adrenergic blocking drugs with (*a*) their myocardial β-adrenergic blocking action, (*b*) chloroform–water partition ratio, (*c*) pK_a, and (*d*) their local anaesthetic properties.

The surface tension of the aqueous solutions was measured with a Fisher Tensiomat instrument (Model 21). Distilled water was used to dissolve the drugs. Solutions ranging from 0 (distilled water alone) to 200 mM were made daily. All determinations were made at 22–25°. Chloroform–water partition ratios and pK_a values for the compounds were obtained from previously published material (Levy, 1968). The technique yielded a mean value of the surface tension for distilled water of 72·7 dynes/cm. Surface tension was measured at least four times for each drug solution. Mean values were plotted *vs* the drug concentration (mM), using distilled water as reference.

The surface tension lowering effects of (±)-INPEA, (±)-MJ-1999 {4-[1-hydroxy-2′-(isopropylamino)ethyl]methane sulphonanilide hydrochloride}, (+)-INPEA, (±)-Kö-592 [1-(isopropylamino)-3-(*m*-tolyloxy)-2-propanol hydrochloride], (±)-pronethalol and (±)-propranolol are seen in Fig. 1. Table 1 relates this effect to other physicochemical and pharmacological actions. Several features are apparent from these data. (i) The highly lipid soluble agent (±)-propranolol produced the greatest lowering of surface tension, and the most hydrophilic compound (±)-MJ-1999 had the least effect, but the similar surface tension effects produced by (±)-Kö-592 and (±)-pronethalol rule out a conclusion that there is a simple relation between hydrophilicity and surface tension effects. Thus, Kö-592 has a chloroform–water partition ratio of 0·096 while pronethalol has a ratio of 15·0. INPEA, which has a surface tension lowering effect between that of MJ-1999 and pronethalol and Kö-592, has a

TABLE 1. SURFACE TENSION LOWERING EFFECTS OF β-BLOCKERS: RELATION TO PHYSICOCHEMICAL AND PHARMACOLOGICAL PROPERTIES

Compound	$\Delta\eta$[a] (dynes/cm)	$CHCl_3/H_2O$[b]	pK_a[b]	Relative[c]	
				Local anaesthesia (Cornea)	β-Blocking (Atrium)
(±)-Propranolol ..	−22·0	34·5	9·45	1·00	1·00
(±)-Pronethalol ..	−15·4	15·0	9·42	0·61	0·15
(±)-Kö-592	−12·9	0·096	8·57	0·57	1·19
(±)-INPEA	−5·9	1·29	8·82	0	0·04
(+)-INPEA	−3·4	1·29	8·78	0	0·002
(±)-MJ-1999 ..	0	0·03	8·26–9·89[d]	0	0·09

[a] Change in surface tension (dynes/cm) produced by 100 mM drug concentration (relative to water)
[b] Values taken from Levy (1968).
[c] Data from Levy (1968). Propranolol = 1·00.
[d] pK_a = 8·26 (ionization of sulphonamide group).
pK_a = 9·89 (ionization of amine HCl).

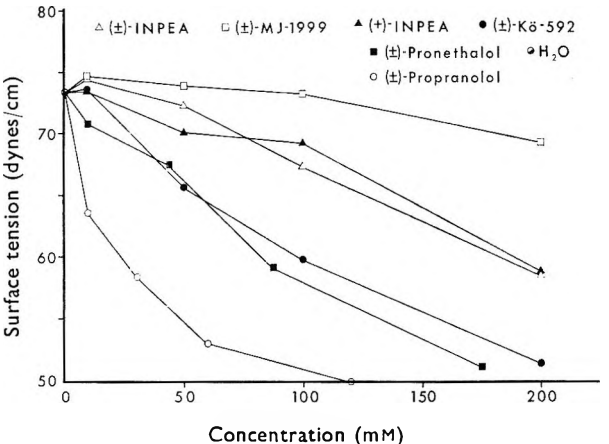

FIG. 1. Surface tension lowering effects of various β-adrenergic receptor blocking drugs.

chloroform–water ratio of 1·29. (ii) Optical isomerism apparently does not modify the surface tension effects of INPEA. (iii) The surface tension effect of these drugs is apparently unrelated to their *in vitro* myocardial β-adrenergic blocking potency. (iv) There is some parallelism between local anaesthetic activity and the ability of these compounds to lower surface tension. This is particularly evident for propranolol. Pronethalol and Kö-592, which are approximately equal in their local anaesthetic actions (rabbit cornea test) show similar effects in lowering surface tension. MJ-1999 and INPEA are devoid of local anaesthetic effects. Concentrations required to lower surface tension are many times higher than those producing myocardial β-blockade *in vitro* (Levy, 1968) but are related to concentrations used to demonstrate surface anaesthesia.

No apparent simple correlation exists between surface tension and β-adrenergic blocking potency. As with the chloroform–water partition ratio, there does appear to be some parallelism between surface tension effects and ability to produce local anaesthesia or depress myocardial contraction (Levy, 1968) for these compounds. While it is recognized that lowering surface tension (air–water) may merely signify the degree of non-wettability of the substance in solution, the marked differences in surface activity seen with agents having such diverse pharmacological and physicochemical properties as MJ-1999 and propranolol cannot be discounted. The hydrophilic methanesulphonanilide moiety characteristic of MJ-1999 yields a molecule with very low lipid solubility. An agent such as propranolol, which has a relatively high lipophilicity, may be surface-active by virtue of its ability to interact with the hydrogen-bonded water molecules. The predominantly non-polar characteristics of this molecule may result in it being positively adsorbed to the hydrogen-bonded water molecules with a concomitant orientation at the air–water interface. MJ-1999, with its predominant polar characteristic, would be negatively adsorbed, and as a result would be uniformly dispersed in the solution rather than orientated at the air–water interface.

The pronounced differences between such compounds as MJ-1999 and propranolol may be related to what Skou (1954) called "capillary activities" of local anaesthetic drugs. This property of being able to alter surface tension

activity therefore may be of importance in explaining the wide differences in pharmacological effects produced by β-adrenergic blockers represented by MJ-1999 and propranolol, particularly in their cell membrane actions.

Acknowledgements. This research was supported in part by grants-in-aid from the American Heart Association (66–645) and the National Institutes of Health, U.S.P.H.S. (1–R03–MH15415–01). The author is a recipient of a Research Career Programme Award from the National Heart Institute, N.I.H. (1–K3–HE–4355).

Research Laboratories,
Presbyterian Hospital,
Pacific Medical Center,
San Francisco, California 94115, U.S.A.

JOSEPH V. LEVY

July 2, 1968

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Hypotensive action of ephedrine in cats

SIR,—In the previous report (Maj & Langwiński, 1966) we described a temporary decrease of blood pressure in cats and rats after tachyphylaxis to tyramine. The depressor action of tyramine was regarded as being connected with the process of histamine liberation. Ephedrine belongs to the group of amines which cause tachyphylaxis. The purpose of the present paper was to find whether ephedrine causes a depressor action and if so, its mechanism of action.

The experiments were made on 40 cats anaesthetized with chloralose (80 mg/kg, i.p.) or urethane (1·5 g/kg, i.p.) accompanied by bilateral cervical vagotomy. Blood pressure was recorded from the carotid artery by a mercury manometer, respiration—with Marey's tambour, the kidney volume plethysmographically, and the contraction of the nictitating membrane recorded isotonically. All the substances were injected into a femoral or jugular vein. Ephedrine hydrochloride of specific rotation of $-33°$ to $-35·5°$ was used.

A hypotensive action of ephedrine in doses of 5 and 10 mg/kg was seen after tachyphylaxis was reached. The general dose of ephedrine averaged 8·9 ± 1·9 mg/kg. When such an average dose was administered, the additional dose of 5 mg/kg of ephedrine caused the depression of blood pressure of 14·6 ± 1·4mm Hg and the dose of 10 mg/kg caused the depression of the pressure 21·4 ± 2·3 mm Hg. Such a decreased blood pressure lasted 2–5 min. The depressive action of ephedrine was accompanied by the decrease of the kidney volume. During the hypotensive phase of ephedrine action, the contraction of the nictitating membrane was unchanged.

The type of anaesthesia, vagotomy, atropine (0·5 mg/kg), antazoline (10·0–30·0 mg/kg), cyclizine (2·0 mg/kg), methysergide (0·5–1·0 mg/kg), dichloroisoprenaline (5·0 mg/kg), propanolol (2 mg/kg) did not abolish or decrease the hypotensive action of ephedrine. Dihydroergotamine (1·0–2·0 mg/kg) and phentolamine (0·5–1·0 mg/kg) abolished the hypotensive action and decreased the kidney volume affected by ephedrine (5·0–10·0 mg/kg). Blackwell & Marley (1967) found that the hypotensive action of some amines depends on the values of the blood pressure in rats. Dihydroergotamine and phentolamine depressed the

blood pressure and therefore after their administration, continuous intravenous angiotensin infusion was used to increase the blood pressure to the initial value. In those conditions dihydroergotamine and phentolamine also abolished the hypotensive action of ephedrine. Ganglion blocking agents like chlorisondamine (2 mg/kg) and pempidine (2 mg/kg) only reduced the hypotensive action of ephedrine.

In contrast to tyramine (Maj & Langwiński, 1966), ephedrine in the concentrations used (10^{-5}–10^{-3}) relaxed the isolated jejunum of guinea-pig.

The results of the experiments showed that the depressive responses do not come from the released histamine as is the case with tyramine (Maj & Langwiński, 1966). Paton (1957) found also that ephedrine does not release histamine from the isolated skin preparation of the cat. It is supposed that the depressive action of ephedrine can be explained by the blockade of adrenergic α-receptors because drugs blocking those receptors (dihydroergotamine and phentolamine) abolished the hypotension observed after ephedrine. This supposition is supported by the reports of Valette, Cohen & Huidobro (1960), who discovered that the continuous infusion of noradrenaline in dogs caused the depressive action of the second dose of ephedrine (3 mg/kg) and also by the explanation of Fleckenstein & Burn (1953) who found that ephedrine besides its indirect effect also possesses direct activity.

Acknowledgement. The authors express their grateful appreciation to Professor Zdzislaw Kleinrok for his kind help during preparation of this study.

Department of Pharmacodynamics,
Medical Academy,
Lublin, Poland
Staszica 4

R. Langwiński
E. Jagiełło

July 12, 1968

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Journal of Pharmacy and Pharmacology

OCTOBER 1968 **VOL. 20 No. 10**

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Letters to the Editor

Printed by W Heffer & Sons Ltd Cambridge England