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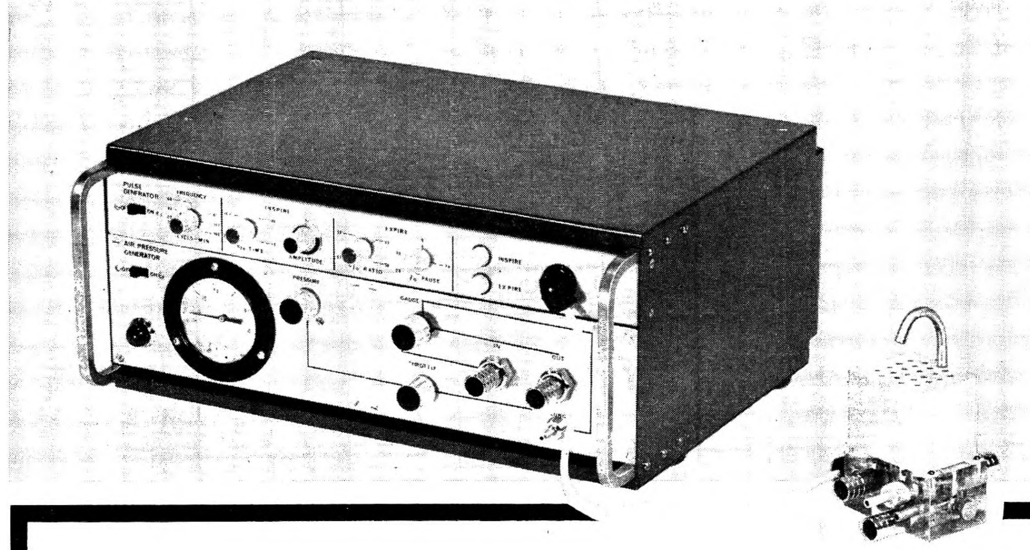

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Cell culture as a test system for toxicity

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Appropriate cell culture systems provide a useful additional method of screening for toxicity, in spite of the obvious problems of relating *in vitro* effects of test compounds at the cellular level to their effects in the whole animal. It is shown that a wide range of chemically disimilar molecules have a reversible inhibitory activity on the growth of primary cultures of monkey kidney cells. The potency of these compounds correlates with their lipid solubility, suggesting that the cell membranes may be their main site of action. Support for this is obtained by the correlation of inhibitory activity and the ability of the same compounds to stabilize the erythrocyte membrane against hypotonic haemolysis which is known to be a direct effect of interaction with the erythrocyte membrane. It is suggested that the ability of the food additive butylated hydroxytoluene to act as a potent inhibitor of cellular growth may account for its reported ability to prolong the life span of mice.

Toxic effects with a low level of incidence, or associated with long term dosage are often established only after extensive usage. They are not readily detected by the conventional *in vivo* studies and it is recognized (e.g. WHO Report, 1967) that additional methods of assessing toxicity are required.

This paper suggests that *in vitro* toxicity studies of freshly isolated cells in culture can provide a valuable source of additional information. Potential toxicity may be expressed as any significant change in the growth and function of treated cells compared with untreated controls. It is possible to establish the dose-response, time course, and reversibility of any significant changes resulting from treatment of primary cells in culture, and studies of this kind on cell monolayers (Milner, 1967) and liver explants (Kirkby, 1964) have been reported. The advantage of these *in vitro* systems is that they are amenable to wide a range of microscopic and biochemical studies in a short time.

Not every significant change in treated cells need be toxic in the whole animal, but ideally the onus of proof should be to show that changes at the cellular level in culture do not result in toxicity *in vivo*. To illustrate the use of cell culture in this context the effects of a range of lipid-soluble molecules on primary cultures of Rhesus monkey kidney (MK) epithelial cells have been examined. In particular the relation between the metabolic inhibition produced in this *in vitro* system by the food additive butylated hydroxytoluene (BHT), and the *in vivo* effects of the same compound reported recently by Harman (1969) is considered.

METHODS

The experimental methods for culturing this system have been reported elsewhere (Milner, 1967). Briefly, monolayer cultures of MK cells were subcultured and grown in Eagles 199 tissue culture medium containing 10% calf serum and antibiotics. After 24 h growth the cells were exposed to the required concentration of test compound dissolved in growth medium containing 1·0% dimethyl suphoxide (DMSO) or 1·0%

dimethyl formamide (DMF). Some control cultures included 1·0% of either solvent, and others were maintained in growth medium alone. In addition to using solvents, BHT was also dissolved in growth media directly. A solution of the antioxidant was evaporated to dryness on a rotor evaporator to leave a thin film in a round-bottomed flask. This film was then taken up into solution by swirling growth medium in the flask (solubility was enhanced by the presence of calf serum). Cell growth was measured both by mitotic indices and by cell numbers using the method of Eagle & Foley (1958). The rate of synthesis of DNA, RNA and protein was followed by the rate of uptake of radio-labelled precursors.

The effect of test compounds on the haemolysis of erythrocytes was followed by the method of Seeman & Weinstein (1966). Where the compound was first dissolved in DMSO, an equivalent concentration of DMSO was also added to the control erythrocyte suspensions.

RESULTS

Solvent effects

Where drugs and additives are insoluble in water a suitable solvent is required that must not in itself affect cell growth nor alter the properties of the solute. Low doses of DMSO tested *in vivo* and *in vitro* have little or no biological effect (e.g. Brown, 1963; Kligman, 1965; Worthley & Schott, 1966; Hollman, Farrelly & Martin, 1967; and *Ann. N.Y. Acad. Sci.*, 1967)., whereas ethanol was discarded as a possible solvent when it was found that low concentrations (<0·1%) caused an increase in cell growth rate over control cultures. Diamond (1965) found that a large variety of cell cultures grown in the presence of 1·0% DMSO did not differ in growth rate from control cells, and the present results with MK cells are compatible with this. Diamond also showed that DMSO did not alter the molecular organization of hydrocarbons and Dixon, Adamson & others (1965) found no difference between *in vivo* effects of various water soluble drugs dissolved in DMSO or in saline. The possibility that the oxidizing activity of DMSO (Muset & Martin-Esteve, 1965) might affect the antioxidant action of BHT is improbable since BHT was equally potent as an inhibitor of cell growth when dissolved in either DMSO or the non-oxidizing solvent DMF. Finally, the inhibitory effect of BHT added in DMSO solution was indistinguishable from its activity when prepared as a solution directly from a thin film of BHT.

Drug effects

At concentrations up to 30 mg BHT/g cells there were no visible cytopathological effects in the MK cells examined by phase contrast microscopy. However this dose was found to inhibit the rate of MK cell growth as indicated by mitotic indices and cell counts and the inhibition was dose-dependent (Fig. 1). Within 30 min of exposure to BHT there was a decrease in DNA, RNA and protein synthesis, with a similar time course for the decrease of each component (Fig. 2), suggesting that the inhibition of growth by BHT is non-specific in this repect. The inhibition is fully reversible within 1 h and the inhibition and reversal can be repeated several times on the same cells.

A comparison of the effects of analogues of BHT at the same test concentration suggested that the degree of inhibition might be related to the lipid solubility of the analogues, since the more lipid-soluble analogues caused a greater degree of inhibition

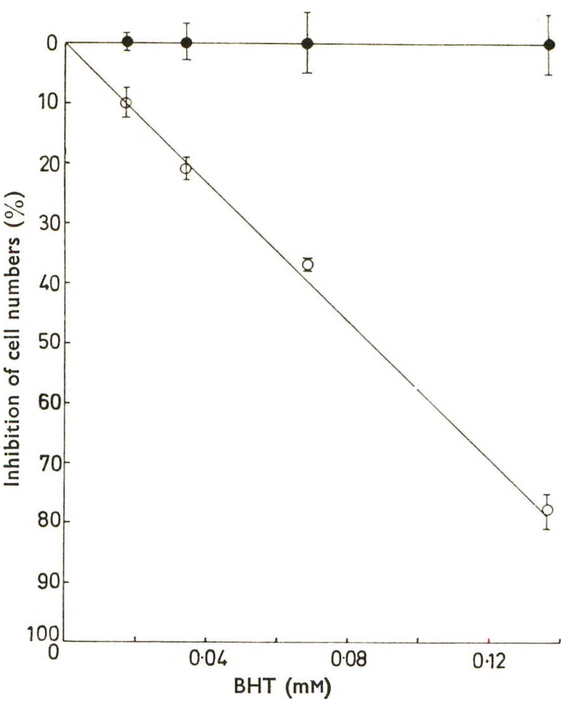

FIG. 1. ○ The dose-dependent inhibition of MK cell growth caused by BHT dissolved in growth medium containing 1·0% DMSO. The percentage inhibition is calculated by the method of Eagle & Foley (1958). ● The growth of cells exposed to 1·0% DMSO alone did not differ from control cultures.

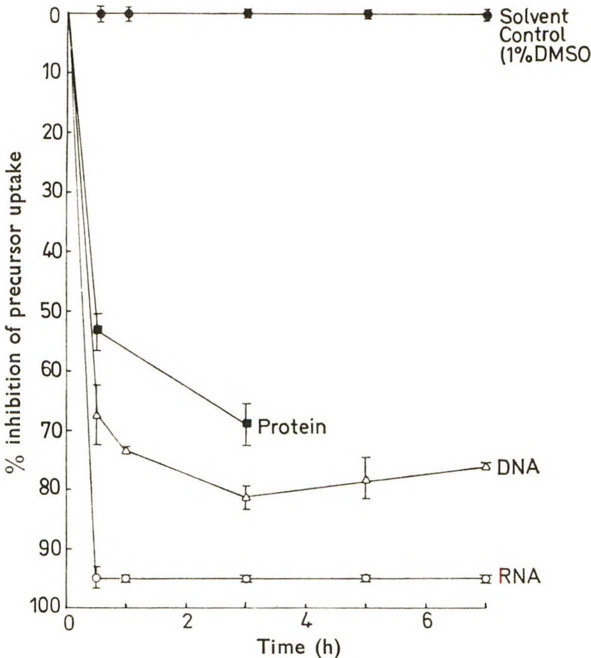

FIG. 2. The inhibition of radiolabelled precursor incorporated into cellular components in the presence of 0·136 mM BHT. ■ [^{14}C]Leucine incorporation into protein; △ [^{3}H]thymidine incorporation into DNA; ○ [^{3}H]uridine incorporation into RNA. ● Cells grown in the presence of 1·0% DMSO had unaltered rates of protein, DNA and RNA synthesis compared with controls.

Table 1. *The relation between the percentage inhibition of MK cell RNA synthesis and the lipid-solubilities of a range of BHT analogues.* The test concentration of each drug was 0·136 mM and inhibition was measured after 3 h. The lipid solubility (π) is expressed as $\log_{10}$ of the partition coefficient relative to phenol (Gilbert & others, 1967).

	I	II	III	IV
% Inhibition of RNA synthesis	0	35	58	70
Lipid solubility π	1·88	3·66	3·98	5·00

I 2-t-butyl-4-methoxyphenol.
II 2,6-di-t-butylphenol.
III 2,6-di-t-butyl-4-methylphenol.
IV 2,4,6-tri-t-butylphenol.

(Table 1). In fact it has been possible to show that a wide range of chemically dissimilar structures produce the same type of metabolic inhibition as BHT at concentrations related to their lipid solubilities (Metcalfe & Metcalfe, 1971).

Taken together these results support the conclusion that the inhibitory effect is caused by a non-specific mechanism which depends primarily on the concentration of the perturbing agent within a hydrophobic environment, and hence on the lipid solubility of the agent. An obvious candidate for the site of localization is the cell membranes, and I have taken as a working hypothesis that cellular metabolism is depressed by structural perturbation of the cell membranes in the presence of extraneous molecules. Perturbation may then result in decreased permeability of the membrane to essential metabolites. It is difficult to rigorously exclude specific sites of interaction as the effective inhibitory mechanism, but this is extremely unlikely in view of the diverse range of chemical structures producing the effect. The data are analogous to the relation of anaesthetic potency to oil/water partition coefficients demonstrated for anaesthetics by Overton and Meyer. More recently Seeman (1966) has produced evidence which strongly supports this relation in a model system. He has demonstrated that the concentration of agents required to stabilize the erythrocyte membrane against hypotonic haemolysis is linearly related to their anaesthetic potency over a concentration range of a million fold. This led me to make an analogous study of the relation of metabolic inhibition in MK cells to erythrocyte stabilization. For the limited number of compounds tested I find that there is a linear relation between MK cell inhibition and erythrocyte stabilization (Fig. 3). The strong inference is that metabolic inhibition is also determined to a good approximation by the concentration of the agent in the membrane and is not greatly dependent on the chemical nature.

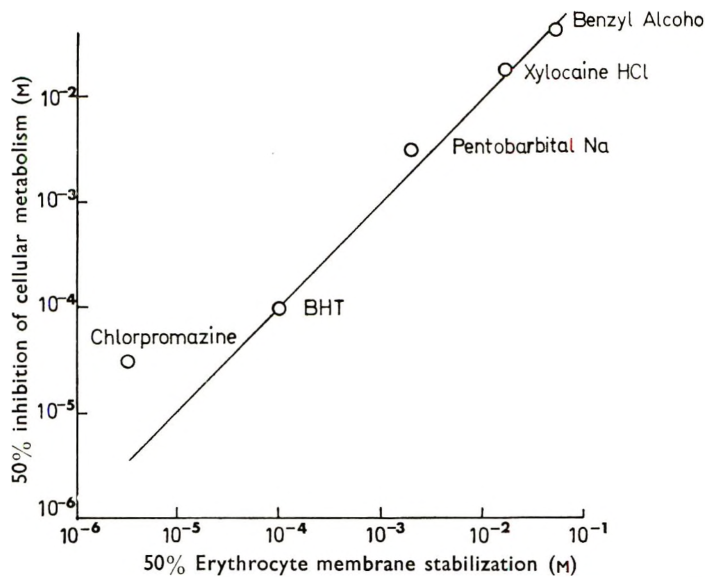

FIG. 3. The relation between metabolic inhibition of MK cells and the stabilization of the erythrocyte membrane against hypotonic haemolysis by a range of chemically dissimilar drugs. The concentration of drug required to produce 50% inhibition of RNA synthesis in MK cells is taken as the inhibitory activity, and is plotted against the concentration of the same drug required to produce 50% stabilization of the erythrocyte membrane (Seeman, 1966).

DISCUSSION

The mechanism of metabolic inhibition caused by lipid-soluble agents is discussed in detail elsewhere (Metcalfe & Metcalfe, 1971). Here, the usefulness of cell culture systems as a screening test for toxicity is considered. The main conclusion is that it is possible in cell culture systems to characterize effects of chemical agents that are not easily detected or analysed *in vivo*. By simple biochemical techniques the type of interaction involved is defined by the absence of chemical specificity, and the cell membrane can be implicated as the site of action. The obvious limitation of the technique is that there is no immediate reason to suppose that the inhibitory effects described for the cell culture system imply toxicity in the whole animal. This is to be contrasted with those specific interactions of drugs with particular subcellular components which may lead to well-defined toxic effects both *in vivo* and *in vitro* (e.g. aflatoxin, heavy metals, fluoroacetate etc.). In practical terms, however, these cases are clear-cut and cell culture experiments simply serve to confirm and define more precisely the causes of acute toxicity.

For this reason it is worth considering the relation of the cell inhibitory effects of BHT to the various *in vivo* studies of the food additive. Extensive evidence supports the safety of BHT at appropriate dose levels (Gilbert & Goldberg, 1965; Ladomery, Ryan & Wright, 1967; Frawley, Kay & Calandra, 1965; Daniel, Gage & Jones, 1968; Clegg, 1965). A report by Johnson (1965) indicated that a dietary level of 0·5% BHT (w/w) reduced the mean weight of offspring of mice, but disputes earlier work by Brown, Johnson & O'Halloran (1959) which suggested that BHT was teratogenic in mice.

It is at least plausible that long term changes in cellular metabolic rates could lead to significant changes in overall physiological function; the changes of course need not be

adverse. In this context it is of interest that Harman(1969) reported that the life span of mice was increased by 44% by the addition of BHT to their diet. It was suggested that the antioxidant properties of BHT might be implicated in the prevention of "cellular deterioration" associated with aging. The dose required per day to produce this effect was 0·5% of the weight of the animal. This is very approximately the equivalent of a dose of 20 mg BHT/g cells in the cell culture experiment. In other words the dose required for metabolic inhibition *in vitro* is of the same order of magnitude as the dose required to produce the *in vivo* effect. The immediate question, which cannot be answered directly from the data available, is whether the two effects are in any way causally related. The cell culture experiments offer as a hypothesis that a general reduction in metabolic rate might be associated with an increased life span. This is susceptible to experimental test, since it would be expected that other (non-toxic) compounds with inhibitory activity would have similar *in vivo* effects. It should also be possible to establish whether antioxidant activity is necessary to increase the life span. Many of the compounds which inhibit MK cell metabolism have little or no antioxidant activity. On the other hand the *in vivo* effect might depend on both metabolic inhibition and antioxidant activity.

Data for the induction in rates of membrane-bound drug metabolizing enzymes by analogues of BHT (Gilbert & Goldberg, 1967) do show a direct relation with the *in vitro* effect, since the more potent enzyme inducers are also the more lipid-soluble analogues. This suggests that enzyme induction is also dependent on the concentration of drug in the membrane, although little is known about the induction mechanism.

These examples of the relation of a well-defined *in vitro* effect to the possible *in vivo* consequences illustrate the potential usefulness of the system. Obviously the choice of cell type is important. Although many workers have used cell lines or cell strains to test foreign compounds *in vitro* (e.g. Gabliks, Bantug-Jurilla & Friedman, 1965) the use of freshly isolated primary cultures for toxicity testing is essential since these often retain the ability to respond to agents that are active *in vivo* (e.g. Milner & Villee, 1970; Michaelides & Coons, 1963; Gillette & Goulian, 1966; Nebert, 1970). This minimizes the problems of alteration in cellular characteristics associated with prolonged culture (Eagle, 1965). It is also preferable to choose cells from the tissue most likely to be affected *in vivo*. For example it is possible that during lipid absorption the rate of cell division in the small intestine may be inhibited by BHT where the epithelial cell lining of the small intestine has perhaps the fastest rate of cell turnover in the body (Creamer, 1967). Hepatic cells are an obvious choice as they are often the most sensitive to toxic action resulting from the accumulation of foreign compounds in the liver. Techniques for maintaining hepatic cells are becoming simpler (Kirkby, 1964; Zuckerman, Tsiquaye & Fulton, 1967) and it may soon be feasible to perform analogous experiments on primary hepatic cell cultures.

Clearly a major problem of *in vivo* testing is the test animal, and the monkey kidney cells used here have the advantage of belonging to the same group of primates as man. This may be preferable to the more common use of rodents, dogs and pigs for metabolic studies *in vivo*. Ultimately it should prove possible to investigate tissue-specific responses by the appropriate choice of a range of cell culture systems.

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Some effects of caffeine and aminophylline on the turnover of catecholamines in the brain

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The effect of caffeine and aminophylline on the turnover of noradrenaline and dopamine in the mouse brain was studied by two different methods: estimation of the rate of disappearance of the transmitter after inhibition of synthesis and measurement of the accumulation of [^{3}H]noradrenaline (^{3}H-NA) and [^{3}H]dopamine (^{3}H-DA) after administration of [^{3}H]tyrosine. After inhibition of the tyrosine hydroxylase the xanthines had little or no effect on the disappearance of both catecholamines. After inhibition of dopamine β-hydroxylase, both xanthines increased the rate of disappearance of noradrenaline. When given in sufficiently large doses both xanthines increased the yield of ^{3}H-NA and ^{3}H-DA from [^{3}H]tyrosine. Pretreatment with a monoamine oxidase inhibitor caused a decrease in the yield of the tritiated catecholamines, which could be counteracted by the xanthines. Stimulation of the noradrenaline receptors by clonidine appeared to cause a decrease in the yield of ^{3}H-NA and an increase in the amount of ^{3}H-DA formed from [^{3}H]tyrosine. Conversely, stimulation of the dopamine receptors by apomorphine caused a decrease in the yield of ^{3}H-DA and an increase in that of ^{3}H-NA. Also, in caffeine-treated animals, clonidine and apomorphine decreased the yield of ^{3}H-NA and ^{3}H-DA respectively. However clonidine could not increase ^{3}H-DA concentrations, nor apomorphine the ^{3}H-NA concentrations more than did caffeine alone. Thus, caffeine and aminophylline appear to increase the rate of turnover of both catecholamines in the brain.

Methylxanthines and especially caffeine are ingested daily by most people in coffee, tea and chocolate. As these compounds exhibit a wide range of pharmacological activities (see Goodman & Gilman, 1970) their possible interaction with drugs cannot be ignored. To predict and reveal such interactions, a detailed knowledge of the mode of action of the methylxanthines is essential.

Recently theophylline and caffeine were shown to increase the rate of synthesis of noradrenaline in the brain, as revealed by the use of labelled tyrosine, and to increase the rate of its disappearance after tyrosine hydroxylase inhibition. The two methylxanthines were also able to potentiate the accumulation of noradrenaline in the brain after monoamine oxidase (MAO) inhibition (Berkowitz, Tarver & Spector, 1970).

The present investigation confirms and extends these observations. In addition to a tyrosine hydroxylase inhibitor, a dopamine β-hydroxylase inhibitor, FLA-63 (Carlsson, Corrodi & others, 1970; Florvall & Corrodi, 1970), was used. Moreover, brain dopamine concentrations were also recorded. In an attempt to analyse the action of caffeine on the turnover of catecholamines, clonidine and apomorphine were used to stimulate chemically the noradrenaline and dopamine receptors respectively (Andén, Rubenson & others, 1967; Andén, Corrodi & others, 1970).

METHODS

Female mice, about 20 g were used. Noradrenaline and dopamine were determined spectrophotofluorimetrically, after tissue extraction in perchloric acid and subsequent strong cation exchange chromatography on Dowex 50 columns (Bertler, Carlsson & Rosengren, 1958; Carlsson & Waldeck, 1958; Carlsson & Lindqvist, 1962). [^{3}H]Noradrenaline (^{3}H-NA) and [^{3}H]dopamine (^{3}H-DA) were determined by liquid scintillation counting, after separation on alumina and Dowex columns (Persson & Waldeck, 1968).

Drugs used were: Apomorphine chloride, caffeine, clonidine, α-methyltyrosine methylester (H 44/68), bis-(4-methyl-1-homopiperazinylthiocarbonyl)-disulphide (FLA-63), nialamide and aminophylline. L-Tyrosine-ring-[3,5-^{3}H] was obtained from NEN Chemicals, Dreieichenhein, and from The Radiochemical Centre, Amersham.

The data shown in Table 3 are derived from two experimental series using [^{3}H]-tyrosine from different sources. This caused differences in the yield of [^{3}H]catecholamines which could be compensated for by using the pxq factorial test of Winer (1962).

RESULTS

Effect of caffeine and aminophylline on the disappearance of brain catecholamines after inhibition of their synthesis

Caffeine or aminophylline (50 mg/kg) were given intraperitoneally alone, or together with either of the synthesis inhibitors H 44/68 (200 mg/kg), or FLA-63 (40 mg/kg). Animals receiving either of the synthesis inhibitors alone were run in parallel. Untreated animals served as controls. Two h after the administration of the drugs the animals were killed and the amount of noradrenaline in the brain was determined.

Caffeine had no effect on the concentration of brain noradrenaline (Table 1), whereas aminophylline appeared to cause a slight increase ($P < 0{\cdot}05$). Neither of the two xanthines significantly changed the extent of noradrenaline depletion brought about by H 44/68. After FLA-63, however, noradrenaline disappeared much faster both when caffeine and when aminophylline were given with the inhibitor ($P < 0{\cdot}05$ and 0·025 respectively).

Table 1. *Effects of caffeine and aminophylline on the disappearance of noradrenaline from the mouse brain after inhibition of its synthesis.* Caffeine (50 mg/kg), aminophylline (50 mg/kg), the tyrosine hydroxylase inhibitor H 44/68 (200 mg/kg) and the dopamine β-hydroxylase inhibitor FLA-63 (40 mg/kg) were given intraperitoneally to mice in the combinations shown below. Two h later the animals were killed and the amount of noradrenaline in the brain was determined. Untreated animals served as controls. Shown are the means ± s.e. as in per cent of the control value. Figures in parentheses denote the number of experimental groups, each comprising six animals.

	Synthesis inhibitor		
Drug	None	H 44/68	FLA-63
None	100 ± 4 (5)	62 ± 5 (7)	45 ± 3 (5)
Caffeine ..	98 ± 11 (2)	69 ± 4 (4)	27 ± 1 (2)
Aminophylline	114 ± 2 (4)	67 ± 3 (4)	29 ± 2 (4)

Table 2. *Effects of caffeine and aminophylline on the disappearance of noradrenaline and dopamine from the mouse brain following tyrosine hydroxylase inhibition by H* 44/68. Mice received H 44/68 (200 mg/kg, i.p.) alone, or followed 1 h later by caffeine (50 mg/kg, i.p.) or aminophylline (50 mg/kg, i.p.). Three h after the administration of H 44/68 the animals were killed and catecholamine contents of the brain were determined. Untreated animals served as controls. Shown are the means ± s.e. in per cent of the control value. Figures in parentheses denote the number of experimental groups each comprising six animals.

Control	H 44/68	H 44/68 + caffeine	H 44/68 + aminophylline
	Noradrenaline		
100 ± 1 (4)	65 ± 4 (4)	62 ± 2 (4)	50 ± 3 (3)
	Dopamine		
100 ± 3 (4)	37 ± 1 (4)	45 ± 5 (4)	43 ± 9 (4)

In the next experiment H 44/68 was given 1 h before the xanthines, the animals being killed 3 h after the administration of the inhibitor. Other experimental conditions were the same as above. The concentrations of both catecholamines in the brain were determined (Table 2). In this case the disappearance of noradrenaline after H 44/68 was accelerated by aminophylline ($P < 0{\cdot}005$), whereas caffeine had no effect. The disappearance of dopamine after H 44/68 appeared to be diminished by the xan thines, but this could not be verified statistically due to a large scatter in the groups with the combined treatment.

Effect of caffeine and aminophylline on the yield of ^{3}*H-NA and* ^{3}*H-DA from* [^{3}*H*]*tyrosine*

[^{3}H]Tyrosine (5 μg/kg) was given intravenously to mice. Some animals were given caffeine (50 or 100 mg/kg, i.p.) 30 min or 2 h beforehand, whilst others received aminophylline in the same doses and at the same time intervals. One h after the administration of [^{3}H]tyrosine the animals were killed and their brains removed and analysed for ^{3}H-NA and ^{3}H-DA.

The results in Table 3 show that the lower dose of caffeine did not change the amount of ^{3}H-NA in the brain at any time interval, whereas when given 2 h before [^{2}H]tyrosine it significantly reduced that of ^{3}H-DA ($P < 0{\cdot}05$). When 100 mg/kg of caffeine was given 30 min before [^{3}H]tyrosine, the yield of both [^{3}H]catecholamines was increased twofold ($P < 0{\cdot}001$). When given 2 h beforehand there was no effect. Aminophylline (50 mg/kg) when given 30 min but not 2 h before [^{3}H]tyrosine increased the amount of ^{3}H-NA ($P < 0{\cdot}01$). At a dose of 100 mg/kg of the drug, the amount of both ^{3}H-NA and ^{3}H-DA in the brain was significantly increased when given 30 min before ($P < 0{\cdot}001$ and 0·01 respectively), less significantly (^{3}H-NA, $P < 0{\cdot}05$) or not at all (^{3}H-DA) when given 2 h before [^{3}H]tyrosine.

Effect of caffeine and aminophylline on the yield of ^{3}*H-NA and* ^{3}*H-DA from* [^{3}*H*]*tyrosine after MAO inhibition*

Mice received the MAO inhibitor, nialamide (100 mg/kg, i.p.) 17 h and (50 mg/kg, i.p.) 1 h before the administration of [^{3}H]tyrosine (5 μg/kg, i.v.). Some animals received caffeine (50 or 100 mg/kg, i.p.) together with the second injection of nialamide,

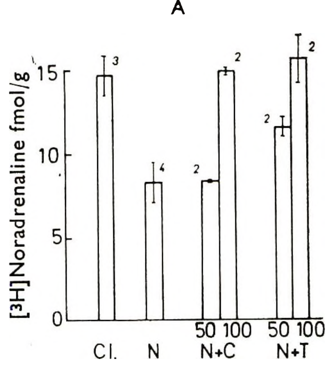
Table 3. *Effects of caffeine and aminophylline on the synthesis of [³H]noradrenaline (³H-NA) and [³H]dopamine (³H-DA) from [³H]tyrosine in the mouse brain.* Mice were given caffeine or aminophylline intraperitoneally 30 min or 2 h before the administration of [³H]tyrosine (5 μg/kg, i.v.). Control animals received [³H]tyrosine alone. One h after the labelled precursor had been given the animals were killed and ³H-NA and ³H-DA in the brain determined. Shown are the means in fmol/g tissue (1 femtomol = 10^{-15} mol). Each mean is based on 4–6 experimental groups. There were six animals per group.

[³H]amine	Control	Caffeine 50 mg/kg		Caffeine 100 mg/kg		Aminophylline 50 mg/kg		Aminophylline 100 mg/kg	
		30 min	2 h	30 min	2 h	30 min	2 h	30 min	2 h
³H-NA	15·2	16·9	13·3	31·1‡	16·2	23·9†	15·8	28·2‡	22·8*
³H-DA	42·5	44·9	29·5*	84·0‡	38·7	54·2	39·8	61·7†	48·1

Significantly different from the control: *$P < 0{\cdot}05$; †$P < 0{\cdot}01$; ‡$P < 0{\cdot}001$; pxq factorial test, see methods.

whilst others received aminophylline in the same doses. One h after the labelled precursor had been given the animals were killed and their brains analysed for ³H-NA and ³H-DA. Mice receiving [³H]tyrosine alone served as controls.

Pretreatment with nialamide reduced the yield of both ³H-NA and ³H-DA by about 40% ($P < 0{\cdot}01$ and 0·001 respectively, see Fig. 1). Given in the high dose, caffeine and aminophylline restored the yield of ³H-NA in nialamide-treated mice to the control value ($P < 0{\cdot}01$) but only aminophylline was able to do the same with the yield of ³H-DA ($P < 0{\cdot}001$). Other combinations gave intermediate values or no effect at all.



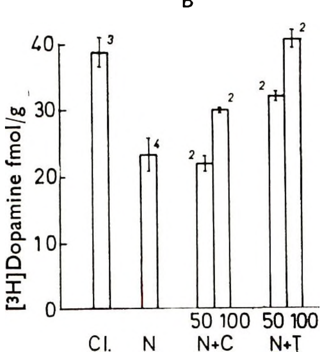


FIG. 1. Effect of caffeine and aminophylline on the synthesis of (A) [³H]noradrenaline (³H-NA) and (B) [³H]dopamine (³H-DA) from [³H]tyrosine in the mouse brain after monoamine oxidase inhibition. Mice were given nialamide (100 mg/kg, i.p.) 17 h and (50 mg/kg, i.p.) 1 h before [³H]tyrosine (5 μg/kg, i.v.). Caffeine or aminophylline was given together with the second injection of nialamide. Controls received [³H]tyrosine alone. The graph shows the amount of ³H-NA and ³H-DA found in the brain 1 h after the labelled precursor had been given. Figures above bars indicate the number of experimental groups (each comprising six animals) from which the mean ± s.e. has been calculated. Cl = control; N = nialamide; C = caffeine, 50 and 100 mg/kg respectively; T = aminophylline, 50 and 100 mg/kg respectively.

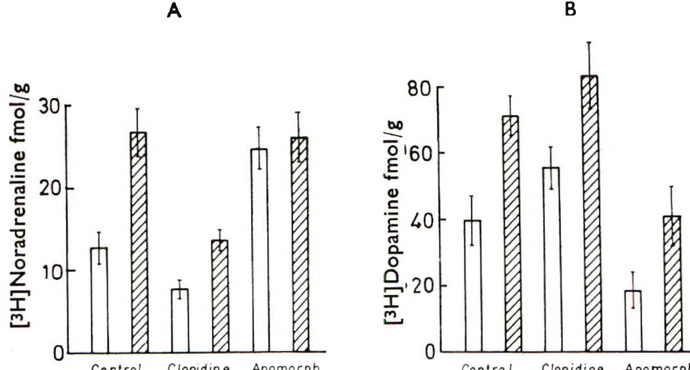


FIG. 2. Effect of caffeine on the synthesis of (A) [^{3}H]noradrenaline and (B) [^{3}H]dopamine from [^{3}H]tyrosine in the mouse brain after chemical stimulation of the receptors. Clonidine (3 mg/kg, i.p.) or apomorphine (25 mg/kg, i.p.) was given alone or together with caffeine (100 mg/kg, i.p.), 30 min before the administration of [^{3}H]tyrosine (5 μg/kg, i.v.). Control animals received [^{3}H]tyrosine alone or in combination with caffeine. The graph shows the amount of [^{3}H]amines found in the brain 30 min after the labelled precursor had been given. The data are the means $\pm$ s.e. of 4–5 experimental groups, each comprising six mice. Shaded columns: caffeine pretreatment.

Effect of caffeine on the yield of ^{3}H-NA and ^{3}H-DA from [^{3}H]tyrosine after chemical stimulation of the catecholamine receptors

Some mice received clonidine (3 mg/kg, i.p.) or apomorphine (25 mg/kg, i.p.) alone, or together with caffeine (100 mg/kg, i.p.). Other animals received caffeine alone. Thirty min later [^{3}H]tyrosine (5 μg/kg, i.v.) was given and after another 30 min the animals were killed and the yield of ^{3}H-NA and ^{3}H-DA in the brain was determined. Controls were given [^{3}H]tyrosine only.

In this experiment also, ^{3}H-NA and ^{3}H-DA in the brain increased after caffeine pretreatment ($P < 0{\cdot}001$ and 0·05 respectively, Fig. 2). After clonidine, ^{3}H-NA tended to decrease whereas ^{3}H-DA if anything increased. However, these changes were not statistically significant. Apomorphine, on the other hand, increased the yield of ^{3}H-NA twofold ($P < 0{\cdot}01$) and reduced the yield of ^{3}H-DA to half the control value ($P < 0{\cdot}001$). The increase in the yield of ^{3}H-NA from [^{3}H]tyrosine brought about by caffeine was antagonized by clonidine ($P < 0{\cdot}01$) but not by apomorphine. In contrast, the increase in ^{3}H-DA brought about by caffeine was antagonized by apomorphine ($P < 0{\cdot}05$) but not by clonidine. In no case was the yield of ^{3}H-NA or ^{3}H-DA higher than after caffeine alone.

DISCUSSION

The measurement of the rate of disappearance of catecholamines after synthesis inhibition is a widely used method of estimating their turnover. The amount of labelled amines accumulating during a short time interval after the administration of labelled tyrosine is another method. Since none of them is quite conclusive (Nybäck & Sedvall, 1970; Persson & Waldeck, 1970a; Wurtman, Anton-Tay & Anton, 1969) both have been used in the present study.

The effect of methylxanthines on the disappearance of brain catecholamines after tyrosine hydroxylase inhibition was slight. Only aminophylline, when given 1 h after H 44/68, increased the rate of disappearance of noradrenaline significantly. The rate of disappearance of dopamine after H 44/68, if anything, decreased after the admini-

stration of the xanthines. When, on the other hand, dopamine β-hydroxylase was inhibited by FLA-63 both xanthines significantly accelerated the disappearance of noradrenaline from the mouse brain. Such a difference in results obtained with the two inhibitors has been observed previously (Persson & Waldeck, 1971) and is thought to be due to an interaction between noradrenaline-containing and dopamine-containing neurons in the brain (Persson & Waldeck, 1970b).

The effects of caffeine and aminophylline on brain noradrenaline after tyrosine hydroxylase inhibition observed by Berkowitz & others (1970) were more pronounced than those reported here. This may in part be due to species differences (rat and guinea-pig as opposed to mouse). However, other causative factors will be discussed below. The slight elevation of the endogenous noradrenaline concentrations observed after aminophylline (Table 1) was probably transient, since it has not been observed by others (cf. Muschol, Kiefer & Lindmar, 1969; Berkowitz & others, 1970).

The experiments on the accumulation of ^{3}H-NA and ^{3}H-DA after [^{3}H]tyrosine indicate an increased turnover of these amines brought about by the xanthines (Table 3). The data also show that 100 mg/kg is a more effective dose than 50 mg/kg and that the effect is of a relatively short duration. This may explain the poor effect noted in the experiments with H 44/68.

When 50 mg/kg caffeine was given 2 h before [^{3}H]tyrosine, the yield of ^{3}H-DA did not increase but rather decreased below the control value. This may indicate a rebound effect which in turn could explain the decreased rate of disappearance of dopamine caused by caffeine in H 44/68-treated animals (Table 2).

Inhibition of MAO results in increased levels of the catecholamines in the brain followed by a reduced turnover (Carlsson, Lindqvist & Magnusson, 1960), probably due to a negative feed-back mechanism for the regulation of catecholamines (Neff & Costa, 1966; Spector & others, 1967). The present experiments, with [^{3}H]tyrosine show that aminophylline, and to a lesser extent also caffeine, were able to increase the yield of ^{3}H-NA and ^{3}H-DA in the brain of nialamide-pretreated animals. This is consistent with the observation that caffeine and theophylline given to animals pre-treated with a MAO inhibitor, will raise the endogenous level of brain noradrenaline more than does the MAO inhibitor alone (Berkowitz & others, 1970).

Stimulation of the noradrenaline receptors by clonidine and of the dopamine receptors by apomorphine causes a reduced turnover of the respective transmitters. Here also, an activation of a negative feed-back mechanism has been proposed (Andén & others, 1967; 1970). Conversely, clonidine appears to increase the turnover of dopamine whereas apomorphine increases the turnover of noradrenaline, possibly due to an interaction between these neuronal systems (Persson & Waldeck, 1970b; Persson, 1970). The present data are compatible with these views.

In animals treated with caffeine, clonidine and apomorphine appeared to reduce the turnover of noradrenaline and dopamine respectively to normal values. On the other hand, apomorphine did not increase the noradrenaline, nor clonidine the dopamine, turnover more than did caffeine alone. The interpretation of this interaction is difficult. The results do not clearly indicate an interference by caffeine with the negative feed-back mechanism for the regulation of catecholamine synthesis.

In conclusion, the experiments with synthesis inhibition and with ^{3}H-NA accumulation indicate an increased turnover of noradrenaline in the brain caused by caffeine and aminophylline when given in sufficiently large doses. Also, the turnover of dopamine appeared to be increased by the xanthines as judged from the accumulation

of ^{3}H-DA. How is this increase brought about? Preliminary data show that caffeine is also able to increase the yield of [^{3}H]catecholamines formed from [^{3}H]dopa (unpublished observations), thus suggesting that the site of action is not on the hydroxylation of tyrosine.

Among the biochemical effects of methylxanthines, their inhibitory action on phosphodiesterase, the enzyme responsible for the catabolism of cyclic AMP (Butcher & Sutherland, 1962), has received particular attention. Cyclic AMP appears to act as a second messenger in several regulatory systems (Sutherland & Rall, 1960; Robinson, Butcher & others, 1968) and seems to be involved in transmitter release mechanisms (see Rasmussen, 1970). Consequently, intraneuronal cyclic AMP may be linked to the regulation of catecholamine turnover, in which case the effect of caffeine and aminophylline on the catecholamines described here would be due to inhibition of the catabolism of cyclic AMP. However, more research is necessary to clarify the situation.

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Some aspects of the metabolism of morphine-*N*-oxide

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After administration of morphine-*N*-oxide (MNO) to rats the opiates appearing in the urine were morphine (61%) and MNO (39%). After administration of morphine, the urinary opiates were morphine (80%) and normorphine (20%). When tacrine was given with morphine the urine also contained MNO (46% of total urinary opiates) and the amount of normorphine was much decreased (to 1%), the remainder being morphine (53%). Tacrine and amiphenazole inhibited demethylation of morphine and codeine by a rat liver fraction *in vitro*. MNO had weak inhibitory activity. Neither MNO nor codeine-*N*-oxide were demethylated *in vitro*.

Woo, Gaff & Fennessy (1968) showed that patients being treated with morphine in combination with either tacrine or amiphenazole excreted in their urine a substance resembling morphine-*N*-oxide (MNO), but this substance was not detected when morphine was administered alone. It was suggested that MNO might be an intermediate metabolite of morphine whose excretion was enhanced in the presence of tacrine or amiphenazole because of inhibition of further metabolism. Fennessy (1968) found that the analgesic activity of MNO was potentiated by tacrine and again suggested that this was due to inhibition of metabolism of MNO. The present investigation deals with the metabolism of morphine and MNO and the effects of tacrine on their metabolism in the rat.

METHODS AND MATERIALS

Drugs

Commercial samples were used of codeine (D.H.A.), morphine sulphate (D.H.A), normorphine (Merck Sharp & Dohme), tacrine (H. W. Woods) and amiphenazole (H. W. Woods). Morphine-*N*-oxide (MNO) and codeine-*N*-oxide (CNO) were synthesized by the method of Freund & Speyer (1910), all reagents employed being of analytical grade.

Administration of drugs

Male albino rats, 300–450 g, in groups of five were injected intraperitoneally with 2 ml of the following solutions: (1) 10 mg/ml of morphine (as the base); (2) 10 mg/ml of morphine (base) plus 1 mg/ml of tacrine; (3) 25 mg/ml MNO; (4) 0·9% (w/v) sodium chloride as control.

Each group was placed in a metabolic cage immediately after injection and the urine was collected for 18 h without contamination by faeces. The receiving vessels contained 1 ml of 11·6 M HCl to prevent bacterial growth.

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Extraction of opiate bases from urine

At the end of the collection period the urine volume was recorded and additional 11·6 M HCl was added to give a final concentration of 1·2 M. The vessel was plugged and autoclaved at 22 p.s.i. ($1{\cdot}52 \times 10^5$ Nm^{-2}) to liberate free bases from conjugates. The bases were extracted into isopropanol–chloroform (1 : 3) after saturation of the aqueous phase with Na_2CO_3 (Milthers, 1961). The MNO, being poorly extracted, was converted to morphine quantitatively by reacting the acidified solution with zinc dust (100 mg) and cupric sulphate (10 μg/ml) for 60 min.

The autoclaved urine samples were divided into two equal portions; one was extracted 3 times with equal volumes of isopropanol–chloroform (1 : 3) to measure excreted morphine and normorphine, the other was similarly extracted after reduction with zinc to measure MNO as additional morphine (a step found not to interfere with the extraction of any morphine or normorphine).

The pooled organic phase extract was evaporated under vacuum in a Büchli Rotavapor to 5 ml. This was quantitatively transferred to a centrifuge tube at 60° and evaporated to dryness by using O_2 free N_2.

The residue was dissolved in 1 ml 0·1 M HCl and interfering substances were removed with 3 washes of n-butanol. An aliquot of 100 μl of the resulting solution was applied with an Agla microsyringe as a broad streak 5 cm in length to a t.l.c. plate of silica gel G (Merck) previously activated at 100° for 30 min. Another 5 μl was applied as a spot together with marker spots.

When the isopropanol–chloroform (1 : 3) solvent front was 17 cm from the origin, the plates were rapidly dried at 50° and redeveloped in the same direction using the same solvent system to provide adequate resolution of morphine, normorphine and MNO.

The plates were then dried and the centre section containing the two 100 μl streaks was covered with stiff paper leaving only the two 5 μl spots and the marker spots on either side uncovered. These were sprayed with iodoplatinate reagent and the plate was examined under ultraviolet light. The areas of the 100 μl streaks corresponding to the fluorescent iodoplatinate spots in the 5 μl urine sections were scraped into centrifuge tubes and those areas not already removed which corresponded to the marker spots of morphine, normorphine and MNO were collected. Background adsorbance was estimated from blank areas of the same plate. The scrapings in each tube were extracted with 5 ml of 0·1 M HCl. After centrifugation at 300 rev/min for 10 min to pack the gel, absorbance of the supernatant solution was measured at 285 nm to estimate opiates.

Preparation of rat liver

Rats were killed by a blow on the head. The liver was rapidly removed, weighed, cooled on ice, and homogenized in ice-cold 0·1 M phosphate buffer at pH 7·4. The homogenate was diluted with phosphate buffer to a final concentration of 500 mg liver/ml and centrifuged at 9000 rev/min for 20 min at 0°. The supernatant containing microsomes and soluble fraction was collected and kept at 0° until incubation.

Demethylating activity of the liver fraction

The demethylating activity of the microsomal + soluble fraction was determined by

a modification of the method of Axelrod (1956), in which formaldehyde production is measured.

The incubation mixtures had the following composition: liver microsomal + soluble fraction (corresponding to 500 mg liver/ml), 0·2 ml; substrate 10 mM, 1 ml; inhibitor 10 mM, 1 ml; NADPH regenerating system 1 ml (NADP 0·2 mM, trisodium isocitrate 15 mM, pig heart isocitrate dehydrogenase (Sigma) 40 μg protein/ml); buffer solution 1 ml (Mg Cl_2, 25 mM, and nicotinamide, 50 mM in 0·5 M phosphate buffer at pH 7·4); Nash B solution (Nash, 1953), 1 ml; water, to make 6·2 ml. The activity of the NADPH regenerating system was checked and found to be satisfactory before use; all mixtures were incubated at 37° with constant shaking.

After incubating for 120 min, the reaction was stopped by adding 2 ml of 40% trichloroacetic acid and the mixture was centrifuged at 3000 rev/min for 10 min. The adsorbance of the supernatant was measured at 412 nm to estimate formaldehyde produced by demethylation of the substrate.

A standard curve using monomethylol dimethyl hydantoin (0·03–0·3 μmol) to replace the substrate in the incubation mixture was prepared daily.

RESULTS

Excretion of opiates in the urine after administration of MNO to rats

Thin-layer chromatographs of the organic extract of both reduced and unreduced samples of rat urine contained a single spot with an R_F of 0·29 corresponding to that of morphine. This was extracted from an unreduced sample and was positively identified as morphine by mass spectrographic analysis; both the m/e peak positions and the disintegration pattern were distinguishable from those expected for MNO, and agreed with the published mass spectrographic data for morphine (Audier, Fetizon & others, 1965).

Normorphine was not detected, but taking into account the sensitivity of the detection method and extraction procedure, less than 70 μg of normorphine, accounting for 0·2% of excreted metabolites, could be present in the urine.

As pointed out in Methods, the difference in the morphine content of reduced and unreduced samples was equivalent to the MNO content. Table 1 shows that 61% of opiates collected in the urine was accounted for as morphine while the remainder was excreted as unchanged MNO.

Table 1. *Total excretion of morphine, normorphine and MNO in urine of a group of 5 rats 18 h after injection of 50 mg MNO to each rat.*

	Morphine	Normorphine	MNO
Concentration of opiate in urine (μg/ml)	382	—	246
Total of each opiate excreted (mg) ..	15·2	0	9·8
Proportion of each opiate in urine ..	61%	<0·2%	39%

The effect of tacrine on the excretion of morphine metabolites in the urine.

Thin-layer chromatograms of the extracts of urine from rats treated with morphine contained two spots the R_F values of which coincided with those of authentic morphine

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Table 2. *Total excretion of morphine, normorphine and MNO in the urine of a group of 5 rats 18 h after intraperitoneal injection of 20 mg morphine or 20 mg morphine plus 2 mg tacrine to each rat.*

	Morphine alone			Morphine plus tacrine		
	Morphine	Normorphine	MNO	Morphine	Normorphine	MNO
Concentration of opiate in urine (μg/ml)	318	82	—	260	10·2	254
Total of each opiate excreted (mg)	2·21	0·54	0	5·20	0·09	4·43
Proportion of each opiate in urine	80·4%	19·6%	<0·1%	53·5%	0·9%	45·6%

(R_F 0·29) and normorphine (R_F 0·11). No MNO was detected; the sensitivity was 35 μg, which is equivalent to 0·1% of recovered opiates.

When morphine and tacrine were administered together, chromatograms of the unreduced urine extracts contained spots corresponding to morphine and MNO (R_F 0·05) but not to normorphine. The spot with the R_F of 0·05 was extracted from the plate and analysed by mass spectroscopy. The m/e value of the primary peak of 301 and the disintegration pattern were indistinguishable from those of a reference sample of MNO. This spot was not present after reduction of the urine sample.

The amounts of morphine, MNO and normorphine excreted in the urine following administration of morphine, or morphine plus tacrine are shown in Table 2. It can be seen that not only was the excretion of normorphine virtually abolished by the administration of tacrine but that MNO then accounted for a large proportion (45%) of the total opiates excreted. In view of these results it was decided to investigate the effect of tacrine on *N*-demethylation *in vitro*.

Demethylation of opiates in vitro

Axelrod (1956) showed that morphine and other opiates may be demethylated *in vitro* and that the products of the reaction were the corresponding demethylated opiate and formaldehyde. Table 3 shows the amounts of formaldehyde formed from codeine, morphine, codeine-*N*-oxide and MNO when these opiates were incubated with a rat liver microsomal + soluble fraction. Codeine was found to be the most reactive substrate; the amount of formaldehyde was barely detectable when morphine was used as the substrate, and no formaldehyde was detected when either codeine-*N*-oxide or MNO were used as substrates.

Amiphenazole and tacrine were found to inhibit significantly the formation of formaldehyde when codeine was used as the substrate (Table 3). A weaker inhibition was observed using MNO as inhibitor. Although only small amounts of formaldehyde were formed when normorphine was used as substrate, amiphenazole, tacrine and MNO reduced this amount to below the minimum detectable level.

DISCUSSION

MNO has weak analgesic activity, being less than one-tenth as potent as morphine (Fennessy, 1968). However, the lipid solubility of MNO is low and it seems unlikely that it would enter the brain. Bickel & Weder (1969) found that after the administration of imipramine-*N*-oxide to rats, none could be detected in the brain. After

Table 3. *The formation of formaldehyde by demethylation of opiates after incubation with rat liver microsomal plus soluble fractions.* Formaldehyde was measured spectrophotometrically after 2 h incubation at 37°. Each entry is the mean of at least five determinations.

Substrate	Amount of substrate added (μmol)	Inhibitor	Amount of inhibitor added (μmol)	Mol formaldehyde produced (mean)‡	Standard error
Codeine	10	—	—	0·31	0·04
Codeine	10	Amiphenazole	10	0·08*	0·02
Codeine	10	Tacrine	10	0·07*	0·02
Codeine	10	MNO	10	0·24†	0·04
Morphine	10	—	—	0·08	0·02
Morphine	10	Amiphenazole	10	<0·05	
Morphine	10	Tacrine	10	<0·05	
Morphine	10	MNO	10	<0·05	
Codeine-*N*-oxide ..	10	—	—	<0·05	
Codeine-*N*-oxide ..	10	Amiphenazole	10	<0·05	
Codeine-*N*-oxide ..	10	Tacrine	10	<0·05	
Codeine-*N*-oxide ..	10	MNO	10	<0·05	
Morphine-*N*-oxide (MNO)	10	—	—	<0·05	
MNO	10	Amiphenazole	10	<0·05	
MNO	10	Tacrine	10	<0·05	

* Significantly different from corresponding inhibitor-free incubation mixture $P < 0{\cdot}01$ (*t*-test).
† Significantly different from corresponding inhibitor-free mixture $P < 0{\cdot}05$ (*t*-test).
‡ Each entry is the mean of seven determinations.

administration of MNO to rats, morphine appeared in the urine in an amount equivalent to 61% of the amount of MNO administered. This finding suggests that the analgesic activity of MNO may be due to the morphine produced in the metabolism of MNO rather than to a direct action of MNO itself. Reduction of *N*-oxides to the corresponding tertiary base is an established metabolic pathway for *N*-oxides, many of those that have been studied in detail do not involve the liver microsomal system (Bickel, 1969).

The absence of normorphine in the urine of rats given MNO, even though appreciable quantities of morphine were excreted, might be explained by the inhibitory effect of MNO on demethylation observed *in vitro*.

The excretion of MNO in urine after administration of morphine together with tacrine confirms in rats the work of Woo, Gaff & Fennessy (1968) who showed that MNO was excreted in patients treated with a mixture of morphine and tacrine. Ziegler, Mitchell & Jollow (1969) have also shown that morphine-*N*-oxide can be formed from morphine in a solubilized preparation of pig liver microsomes; this reaction was NADPH- and oxygen-dependent.

Combination of tacrine with morphine led to a marked reduction in normorphine excretion. This effect is probably related to the inhibitory action of tacrine on the demethylating activity of the soluble and microsomal fraction of liver. In addition, the MNO that was formed has inhibiting activity on demethylation.

A number of other tertiary amines have been shown to be metabolized to form *N*-oxides. The best known examples are imipramine (Bickel, 1969), chlorcyclizine (Burns & Phillips, 1966), chlorpromazine (Beckett, 1968), dimethylamine (Ziegler &

Pettitt, 1964), nicotinamide (Chaykin & Block, 1959) and trimethylamine (Baker & Chaykin, 1962).

Our findings and the work of Ziegler & others (1969) indicate that MNO is a normal metabolite of morphine. Presumably, MNO accumulates in the presence of tacrine because tacrine inhibits further metabolism of MNO or it inhibits an alternative pathway of morphine metabolism; for example, glucuronide conjugation or *N*-demethylation.

Reduction was the only metabolic pathway demonstrated for MNO *in vivo*. This was confirmed *in vitro* by the absence of formaldehyde production when MNO was incubated with rat liver microsomes. MNO, being highly polar, may not be able to penetrate into the microsomes which are necessary for its metabolism. However MNO produced within the microsomes might undergo demethylation at a significant rate.

Though the demethylation of morphine and codeine were shown to be inhibited *in vitro* by tacrine, it seems difficult to account for the amount of MNO which is

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Administration of two or more related drugs to investigate the effect of molecular modification and formulation on drug absorption, metabolism and excretion**

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Two or more related drugs of the amphetamine class were simultaneously administered to man under acidic urine control, their urinary excretion being examined by gas-liquid chromatography. This was shown to be a suitable procedure for determining the effect of molecular modification and formulation on drug absorption, metabolism and excretion.

Gas-liquid chromatography (g.l.c.) may be used to separate stimulant amines and others of closely related structure (Beckett, Tucker & Moffat, 1967). Many of these are excreted in the urine either substantially unchanged or as basic metabolites (Beckett & Rowland, 1965a,b; Beckett & Wilkinson, 1965; Beckett & Brookes, 1967; Beckett & Brookes, 1971; Beckett, Brookes & Shenoy, 1969; Beckett, Salmon & Mitchard, 1969). It should thus be possible to compare *under identical conditions* the rates of excretion of these compounds when they are administered simultaneously.

Under conditions of acidic urine to minimize kidney tubular re-absorption of bases, direct comparison of the effect of molecular modifications on drug absorption and excretion is facilitated, provided mutual interference of the drugs on these processes does not occur. The urinary excretion of some such amines (Ia–i) thus administered was investigated.

METHODS

Gas-liquid chromatography

The conditions were described by Beckett, Brookes & Shenoy (1969); identification of the drug and metabolite peaks from urine extracts was made by comparison with authentic samples and acetone, acetyl or propionyl derivatives.

Dosage regimens

The subjects were healthy males (age 25–45) with maintained acidic urine. The drugs were administered orally in aqueous solution, alone and with one or more drugs of the same group; primary amines (Ia–d; dose = 10 mg base); *N*-methyl derivatives (Ie–g; dose = 10 mg base); *N*-ethyl derivatives (Ih, i; 20 mg HCl salt of each).

Phentermine and ethylamphetamine were administered together on separate occasions as formulated products (as the resin bonded drug in gelatin capsules or in

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** This work forms part of a thesis by L. G. Brookes, accepted for the degree of Ph.D. in the University of London, 1968.

R, Me, $—CH_2—C—NHR''$, R′

	R	R′	R″	
(a)	H	H	H	amphetamine
(b)	CF_3	H	H	norfenfluramine
(c)	H	Me	H	phentermine
(d)	H(p-Cl)	Me	H	chlorphentermine
(e)	H	H	Me	methylamphetamine
(f)	CF_3	H	Me	methylnorfenfluramine
(g)	H	Me	Me	mephentermine
(h)	H	H	Et	ethylamphetamine
(i)	CF_3	H	Et	fenfluramine

I

sugar-coated tablets, respectively) with an aqueous solution of the HCl salt of the other drug. The methods for 24 h sample collection, analysis and maintenance of an acidic urine were as described by Beckett, Brookes & Shenoy (1969).

RESULTS AND DISCUSSION

The retention times on analysis of the compounds and their derivatives are given in Table 1. Nicotine had a retention time of 8·5 min in the system used, so its presence in smokers' urine did not interfere.

There was no significant difference in the total urinary excretion of the compounds administered in aqueous solution when given singly or with other drugs, indicating that the drugs did not interfere with absorption, metabolism, distribution and excretion of each other (see Table 2 for urinary excretion of compounds administered).

Since, under acidic conditions of urine, the plasma concentration and rate of urinary excretion of a basic drug are related (Beckett, Salmon & Mitchard, 1969), the kinetic analysis of the absorption and fate of such drugs in man (Beckett, Boyes & Tucker, 1968a, b; Beckett & Tucker, 1968) under comparable conditions is now possible. In addition, oral administration of marker drugs in solution may be used, under identical conditions, to study the effect of formulation on the bio-availability of other drugs.

Urinary excretion profiles of the drug mixtures administered in aqueous solution are given in Figs 1–2A. From these and Table 2, it may be concluded that chemical substitution in the amphetamine molecule results in the following: (a) halogen substitution causes a marked decrease in total urinary excretion of the drug over a given time period, i.e. more extensive metabolism, together with a delay of up to

Table 1. *Retention times of the compounds examined.*

Column	Stationary phase	Operating temp (°C)	Column length (metres)	Retention times (min)								
				A	MA	EA	P	MP	CP	NF	MNF	F
Chromosorb G acid-washed DMCS treated 80–100 mesh	10% KOH 10% Apiezon L	160	1	2·6	3·6	4·4	3·2	5·2	9·0	1·9	2·4	3·0
				Acetone derivative								
				4·5						2·9		
Chromosorb G acid-washed DMCS treated 80–100 mesh	5%KOH 2% Carbowax 20 M.	165	2	Acetyl derivative								
				7·2	5·2	5·0	4·4	3·6	14·6	5·7	4·2	3·3
				Propionyl derivative*								
				20·1	14·6	14·0	12·3	10·1	41·2	15·8	11·8	9·0

* Operating temperature 140°

A = amphetamine; MA = methylamphetamine; EA = ethylamphetamine; P = phentermine; MP = mephentermine: CP = chlorphentermine; NF = norfenfluramine; MNF = methylnorfenfluramine; F = fenfluramine.

Table 2. *The 24 h urinary excretion of phentermine, amphetamine chlorphentermine and norfenfluramine, and of the* N*-ethyl or* N*-methyl derivatives of some of those compounds after oral doses of solutions of the drugs, with maintained acidic urine.*

		% Drug excreted (including any basic metabolite)			
		Following administration of drug mixtures		Following administration of single drug**	
Drug group	Compound	Range of values	Average*	Average	*
Primary amines	Phentermine	71·7–83·6	76·4 (5)	71·8	(6)
	Phentermine-formulated product	43·3, 50·0	46·7 (2)		
	(+)-Amphetamine	49·2–68·4	60·8 (8)	62·7	(9)
	Chlorphentermine	30·0–39·2	35·0 (4)	35·5	(5)
	(±)-Norfenfluramine	22·4–35·5	28·8 (4)	29·3	(6)
Secondary amines (*N*-methyl)	Mephentermine	68·0–72·2	70·0 (4)	71·4	(2)
	(+)-Methylamphetamine	52·5–58·0	55·4 (4)	60·0	(7)
	(+)-Methylnorfenfluramine	28·9–38·6	34·2 (4)	36·4	(2)
Secondary amines (*N*-ethyl)	(+)-Ethylamphetamine			37·7	(5)
	(±)-Ethylamphetamine	45·5–61·3	53·8 (5)	52·6	(4)
	(−)-Ethylamphetamine			78·9	(3)
	(±)-Ethylamphetamine – formulated product	30·2, 37·2	33·7 (2)		
	(±)-Fenfluramine	29·2–40·1	33·5 (8)	32·4	(6)

* Value in parentheses refers to number of subjects for each trial.
** Date reported by Beckett & Rowland (1965a, b), Beckett & Brookes (1967, 1971), Beckett & others, (1969) and Brookes (1968).

5 h in the peak excretion and in the initial appearance of the drug in the urine (cf. also observed for *p*-chloroamphetamine; Beckett, Mitchard & Salmon, unpublished results), and (b) substitution by a second methyl group on the α-carbon atom causes a significant increase in total excretion, i.e. reduces metabolism, possibly due to additional steric hindrance reducing deamination, but has no influence on the rate of absorption.

The effects of side-chain substitution or ring halogenation (or both) on *N*-alkylamphetamines are similar to those occurring for amphetamine. However, *N*-alkylation, while not influencing the rate of absorption, reduces the amount of drug excreted unchanged, i.e. increases metabolism, *N*-ethylation being more effective than *N*-methylation in this respect. These results are in agreement with those predicted from the buccal absorption test (Beckett & Triggs, 1967; Brookes, 1968), although *N*-alkylation has little effect on the urinary excretion of norfenfluramine, the elimination profile of which, being analogous to that of fenfluramine (Fig. 2B), shows a two-phase exponential decline. The initial phase for the first 8–10 h parallels that of the non-halogenated molecule, ethylamphetamine, but the second phase is of significantly longer half-life (Fig. 2B), indicating slow drug release from an undetermined body compartment. A similar two-phase elimination profile for *p*-chloroamphetamine has recently been obtained (Beckett & others, unpublished results). Also, Jun & Triggs (1970) noted, from blood level studies, that chlorphentermine undergoes multi-compartment distribution.

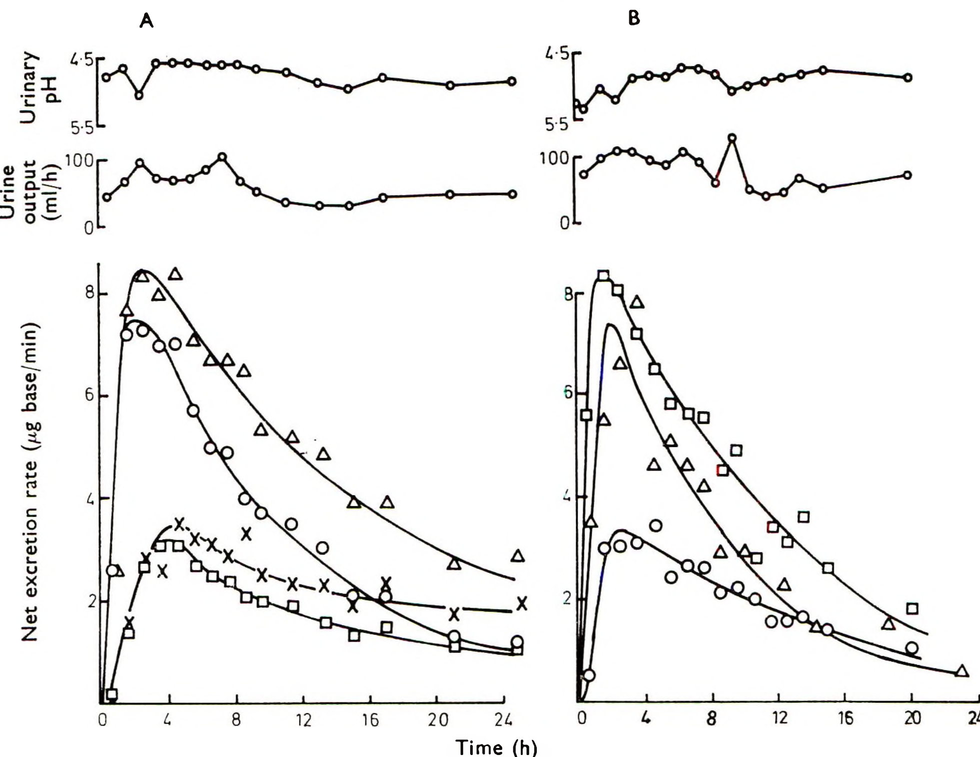

FIG. 1. A. Urinary excretion of amphetamine, phentermine, norfenfluramine and chlorphentermine with corresponding urinary pH and urine output after the simultaneous oral administration (10 mg base of each) of (+) amphetamine (as sulphate), and phentermine, (±)-norfenfluramine and chlorphentermine (as hydrochlorides) to a subject under acidic urine control. —△— Phentermine. —○— Amphetamine. —X— Chlorphentermine. —□— Norfenfluramine.

B. Urinary excretion of mephentermine, methylnorfenfluramine and methylamphetamine, with corresponding urinary pH and urine output, following the simultaneous oral administration (10 mg base of each) of mephentermine sulphate, (±)-methylnorfenfluramine and (+)-methylamphetamine hydrochlorides in aqueous solution to a subject under acidic urine control. —□— Mephentermine. —△— Methylamphetamine. —○— Methylnorfenfluramine.

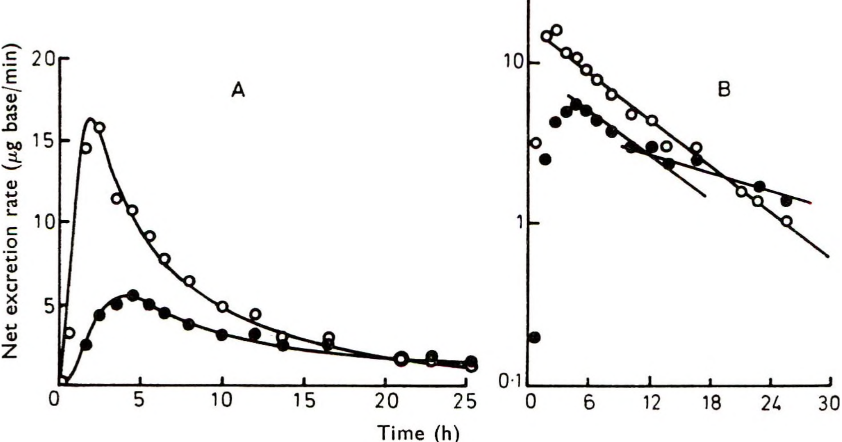

FIG. 2. Urinary excretion of fenfluramine and ethylamphetamine from a subject under acidic urine control, after the simultaneous oral administration of 20 mg each of (±)-fenfluramine (—●—) and (±)-ethylamphetamine (—○—) hydrochlorides in aqueous solution.

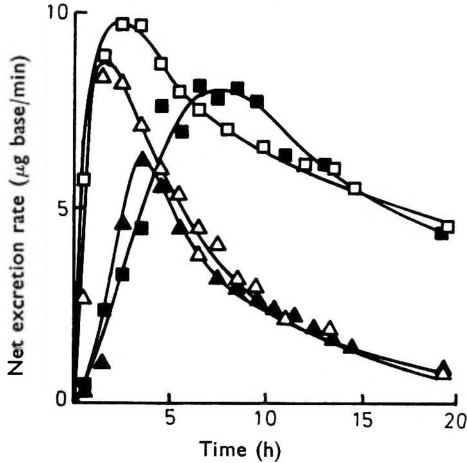

FIG. 3. Urinary excretion of ethylamphetamine and phentermine from a subject under acidic urine control following the oral co-administration of 12 mg (±) ethylamphetamine hydrochloride and 15 mg phentermine in either aqueous solution or formulated product. Dosage regimens: (1) Ethylamphetamine HCl in solution + phentermine capsule. (2) Phentermine in solution + ethylamphetamine HCl tablet. Solutions: —△— ethylamphetamine; —□— phentermine. Formulated products: —▲— ethylamphetamine (sugar-coated tablet); —■— phentermine (hard gelatin capsule containing phentermine-resin complex).

The use of a "marker drug" to illustrate the effects of formulation on the urinary excretion profiles of ethylamphetamine and phentermine is shown in Fig. 3. Resin-bonding with subsequent encapsulation of phentermine, and sugar-coated tableting of ethylamphetamine, delay the time of maximum excretion, and significantly reduce the rate and total amount of drug absorption from the gut, possibly due to the reduced bio-availability of drug caused by incomplete *in vivo* disintegration of the formulated preparations.

Acknowledgements

We are indebted to our colleagues who acted as subjects for this investigation. Companies who provided gifts of the compounds used have been acknowledged previously (Beckett & others, 1967).

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The metabolism and binding of [^{14}C]mycophenolic acid in the rat

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Seventeen h after the intraperitoneal administration of 33 mg/kg of [^{14}C]mycophenolic acid to rats, radioactivity was bound to the tissues of the intestines, bladder, stomach, kidney, liver and lung in decreasing order; no binding to spleen tissue was observed. *In vitro* incubations of the agent with macromolecules resulted in the binding of radioactivity to salmon sperm DNA and to bovine plasma albumin, the extent of binding being increased and decreased, respectively, in the presence of a rat liver microsomal system. The binding was apparently covalent since repeated purification procedures failed to release the bound radioactivity; heating of [^{14}C]mycophenolic acid bound-DNA in N hydrochloric acid at 100° for 2 h caused the release of the bound radioactivity. Under the conditions described, 43% of the administered radioactivity was excreted in the urine (33%) and faeces (10%); the urine contained free mycophenolic acid (13%), mycophenolic acid glucosiduronate (17%) and an uncharacterized metabolite (3%).

Mycophenolic acid (I) a metabolite of several strains of *Penicillium brevi-compactum* (Clutterbuck, Oxford & others, 1932) was first detected by Gosio (1896), isolated and named by Alsberg & Black (1913) and structurally characterized by Raistrick and his co-workers (Clutterbuck & Raistrick, 1933; Birkinshaw, Bracken & others, 1948; Birkinshaw, Raistrick & Ross, 1952). It is an antibiotic (Abraham, 1945; Gilliver, 1946; Brian, 1949) of low toxicity (Carter, Franklin & others, 1969) which also has antimitotic (Carter & others, 1969) and anti-cancer (Carter, 1966; Williams, Lively & others, 1968; Carter & others, 1969) properties; it is also a potent inhibitor of nucleic acid synthesis largely through inhibition of inosinic acid dehydrogenase (Franklin and Cook, 1969; Carter & others, 1969) which converts inosinic acid into xanthylic acid. These properties and possible clinical use of mycophenolic acid against human malignancies seemed to justify an investigation into the metabolism, distribution and tissue binding properties of this agent.

O OR Me
C CH2 CH : C(CH2)2CO2H
O
CH2 OMe
Me

I Mycophenolic acid (R = H) and II mycophenolic acid glucosiduronate (R = $C_6H_9O_6$).

METHODS AND MATERIALS

Radioactive assays were performed on a Packard Tri-carb liquid scintillation spectrometer (Model 3375) and represent counts at least 50% above background corrected for quenching by addition of an internal standard.

Incubations, unless otherwise stated, were performed by shaking the mixtures in a

metabolic shaker (H. Mickle, Gomshall, Surrey) at 65 excursions/min in air at 37°.

Animals. Male 6 week old Chester Beatty rats (150 g) were used for the preparation of liver microsomes or were given intraperitoneal injections of [^{14}C]mycophenolic acid (sp. activity, 0·925 μCi/mg; dose, 30·8 μCi/kg) as a 2% (w/v) solution in polyethylene glycol. In metabolic experiments, the animals were starved for 8 h preceding treatment and for the duration of the experiment; the animals were housed in a polythene cage lined with two sheets of Whatman 3MM filter paper held in position with glass rods. Water was freely available.

Chromatography. Whatman 3MM chromatography paper was used for ascending overnight development in (a) methanol–6N HCl (7:3, by vol) and for descending development in (b) butan-1-ol–acetic acid–water (12:3:5, by vol) and (c) butan-1-ol–propan-1-ol–2M NH_3 (2:1:1, by vol). For t.l.c., glass plates were coated with silica gel G (E. Merck, Darmstadt, W. Germany) of 0·25 mm thickness and the chromatograms were developed in (d) benzene–ethanol (1:1). Mycophenolic acid on chromatograms was detected by ultraviolet light from a chromatolite lamp (Hanovia, Ltd.) and had R_F values 1·0, 0·96, 0·42 and 0·85 in solvents (a) to (d) respectively.

Materials

[^{14}C]Mycophenolic acid (sp. activity, 592 mCi/mol) and mycophenolic acid were kindly supplied by Drs. A. Hayes and T. J. Franklin of Imperial Chemical Industries Ltd., Aldersley Park, Macclesfield, Cheshire, U.K.; the radioactive material was diluted with the unlabelled material as required. DNA (from salmon testes) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; bovine plasma albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.; $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase (from yeast) were from Boehringer Corp. (London) Ltd., London, W.5, U.K.; sulphatase (Taka-diastase, from *Aspergillus oryzae*) was from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, U.K.; β-glucuronidase (Ketodase, from bovine liver) was from Warner-Chilcott, Morris Plains, N.J., U.S.A.; and deoxyribonuclease I (D) from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Washed rat liver microsomes were prepared as described by Booth & Boyland (1964).

RESULTS

Metabolism of [^{14}C]*mycophenolic acid*

[^{14}C]Mycophenolic acid (10 mg, 9·25 μCi) was administered to two rats and the urine and faeces were collected for 17 h. Radioactive assay revealed that the faeces contained approximately 10% of the administered radioactivity. The filter paper, glass rods and inside of the cage were washed with portions (200 ml) of aqueous 50% ethanol until no more radioactivity could be extracted. The washings were combined and evaporated in a vacuum and the residue was dissolved in water (10 ml). Radioactive assays of aliquots (0·5 ml) revealed that approximately 33% of the administered radioactivity was excreted in the urine. Chromatography of aliquots (0·1 ml) of the urine concentrate in solvent (c) revealed three areas of radioactivity centred at R_F 0·07, R_F 0·30 and R_F 0·42 representing 17, 3 and 13%, respectively, of the administered radioactivity (see Fig 1). Aliquots (1·0 ml) of the urine concentrate were diluted with 0·2M phosphate buffer, pH 7·0 (0·2 ml) and incubated for 24 h with or without β-glucuronidase ("Ketodase", 0·2 ml; 1000 Fishmann units) or sulphatase (Taka-diastase, 20 mg). Examination of the incubates by chromatography in solvent

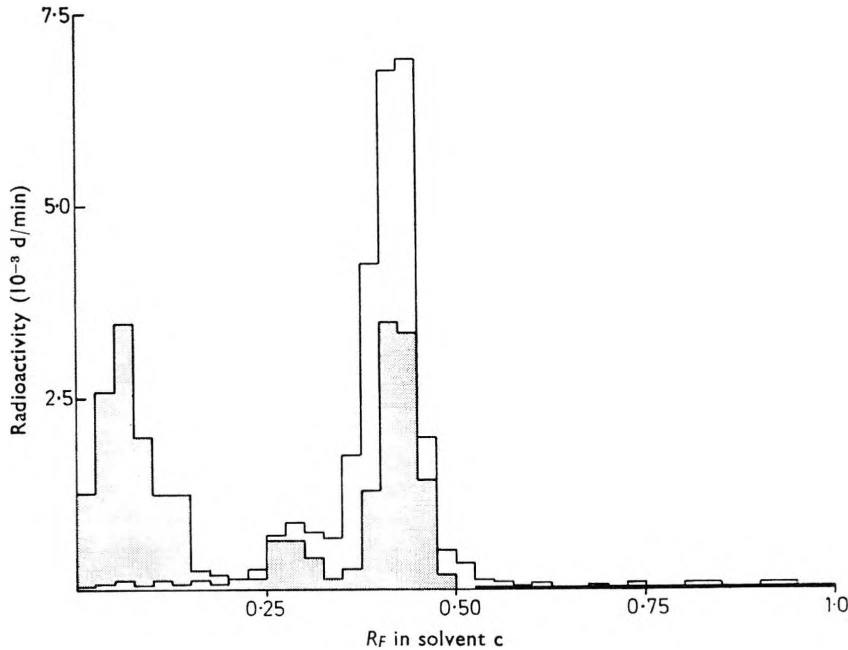

FIG. 1. Histogram of metabolites of [^{14}C]mycophenolic acid excreted in the urine (stippled areas) and of the same after incubation with β-glucuronidase (clear areas). Other details are given in the text.

(c) showed that the radioactivities of the three components in the β-glucuronidase-treated mixture were 1·3, 10·0 and 87·8 % respectively, of the applied radioactivity and were unchanged in the two other mixtures (see Fig. 1).

Aliquots (1 ml) of the urine concentrate, before and after hydrolysis with "Ketodase", were heated with mycophenolic acid (0·4 g) to give clear solutions which, on cooling, deposited colourless prisms, m.p. and mixed m.p. 141°, of mycophenolic acid which was recrystallized from water to constant activity. The results showed that the urine specimen before and after hydrolysis with the enzyme contained 13 and 30 %, respectively, of the administered radioactivity as mycophenolic acid.

The binding of [^{14}C]*mycophenolic acid to rat tissues* in vivo

The rats used in the metabolic experiments described above were killed by cervical dislocation 17 h after administration of [^{14}C]mycophenolic acid. Acetone powders of tissues were prepared and washed repeatedly with acetone until the final washing entrained no radioactivity as determined by scintillation counting. Samples (2 mg) were dissolved in tetraethylammonium hydroxide (0·5 ml) and assayed for radioactivity. The results are shown in Table 1.

The binding of [^{14}C]*mycophenolic acid to salmon sperm DNA and to bovine plasma albumin* in vitro

Duplicate solutions of salmon sperm DNA (30 mg), purified by a detergent-salt procedure (Kay, Simmons & Dounce, 1952) to a residual protein content of less than

Table 1. *Radioactive binding of* [14C]*mycophenolic acid to rat tissue* in vivo *and to DNA and protein* in vitro. [14C]Mycophenolic acid was incubated at pH 7·4 for 1 h at 37° in air with the protein or DNA in the presence or absence of a rat liver microsomal system; or the acid was administered intraperitoneally to rats and acetone powders of the various tissues were prepared 17 h later. Further details are given in the text.

Binding site		Extent of binding*
A. *In vitro*		
Salmon sperm DNA	(i)	0·02, 0·03
	(ii)	0·77, 0·89
Bovine plasma albumin	(i)	2500, 1968
	(ii)	704, 642
B. *In vivo*		
Intestine		1·02
Bladder		0·32
Stomach		0·16
Kidney		0·05
Liver		0·04
Lung		0·02
Spleen		0

* μMol/g atom of DNA-P (assuming 8% P) or per mol of protein (assuming mol wt of 64 000) or per g of acetone powder of tissue. (i) In the absence and (ii) in the presence of rat liver microsomes.

0·2%, or bovine plasma albumin (30 mg) in 1 mM sodium citrate buffer, pH 7·4 (50 ml) was treated with a solution of [14C]mycophenolic acid (0·2 mg, 0·37 μCi) in acetone (0·2 ml). Similar mixtures also contained resuspended washed rat liver microsomes (equivalent to 2 g liver), $NADP^+$ (8 mg), glucose 6-phosphate (38 mg), $MgCl_2$ (15 mg) and glucose 6-phosphate dehydrogenase (0·6 unit). The mixtures were incubated for 1 h; microsomes were removed from the microsome-containing mixtures by ultra-centrifugation at 80 000 *g* for 1 h. The supernatant solutions and the remaining mixtures were extracted with chloroform (3 × 2 ml), the aqueous fractions treated with NaCl (2·5 g) and the nucleic acid or protein was precipitated with ethanol (3 vol) or acetone (3 vol) respectively, washed overnight in fresh solvent and dried in an evacuated desiccator over P_2O_5. The DNA was further purified by a repetition of the detergent-salt procedure and the protein by repeated precipitations with acetone from solution in aqueous −5% (w/v) NaCl until the final supernatant contained no radioactivity. DNA (2 mg) was hydrolysed by incubation in deoxyribonuclease solution (0·5 ml; 7500 units) and protein (2 mg) was dissolved in tetraethylammonium hydroxide (0·5 ml). The radioactivities were determined by scintillation counting. The results are shown in Table 1.

A sample (2 mg, 2534 d/min) of the purified DNA obtained from the DNA-[14C]-mycophenolic acid–microsome mixture was heated in N HCl (1·0 ml) at 100° for 2 h. Chromatography of the hydrolysate (0·1 ml) in solvents (a), (b) or (c) revealed (i) a major area of radioactivity (comprising approx. 93% of the radioactivity) at R_F 0 to R_F 0·1 in solvents (b) and (c) and at R_F 0·31 to R_F 0·54 centred at R_F 0·38 in solvent (a) and (ii) a minor area (comprising the remainder of the radioactivity) at R_F 0·95 to R_F 1·0 in solvents (a) and (b) and at R_F 0·40 to R_F 0·44 in solvent (c); (ii) appeared to be due to mycophenolic acid which showed similar R_F values in the three solvents.

Failure of [^{14}C]*mycophenolic acid to react* in vitro *with purines, pyrimidines and* N-*acetylcysteine*

Solutions of [^{14}C]mycophenolic acid (1 mg, 0·185 μCi) in acetone (0·1 ml) were severally treated with solutions of each of the following substances (2 mg) in 1 mM-citrate buffer, pH 7·0 (0·2 ml): guanine, adenine, cytosine, thymine, uracil and *N*-acetylcysteine. The mixtures were heated at 60° for 4 h and aliquots (0·1 ml) examined by chromatography in solvents (a), (b) and (c). Radioactive assay of strips (2 cm wide by 1 cm along the direction of development) showed that [^{14}C]mycophenolic acid was the only radioactive component of all the mixtures.

DISCUSSION

Administration of [^{14}C]mycophenolic acid into the intraperitoneal cavity of rats resulted in the uneven binding of radioactivity to all tissues examined except the spleen. The highest level of binding occurred in the tissues of the gastrointestinal tract (see Table 1); this result may be related to the observation that mycophenolic acid caused the development of abdominal colic, watery blood-stained diarrhoea and cytotoxic damage to the intestinal mucosa in rhesus monkeys (Carter & others, 1969). Radioactivity from [^{14}C]mycophenolic acid was strongly bound to rat tissues *in vivo* and to salmon sperm DNA and bovine plasma albumin *in vitro*. The binding to DNA was probably covalent since repeated purification procedures failed to release the bound radioactivity; heating of radioactivity bound-DNA at 100° for 2 h in N hydrochloric acid produced a small amount of a radioactive substance chromatographically identical with mycophenolic acid and an unidentified radioactive product having R_F 0·38 in solvent (a). The binding of radioactivity to DNA was apparently potentiated by metabolism since the extent of binding to salmon sperm DNA increased 30 to 37-fold on incubation in the presence of a rat liver microsomal system. The binding to bovine plasma albumin showed the reverse effect (see Table 1); the reason for this is not clear but may be due to competitive binding of radioactivity to the microsomes. The relatively higher binding to bladder than to kidney may indicate hydrolysis of mycophenolic acid β-glucosiduronate by the β-glucuronidase of urine; this indicates that at least part of the free mycophenolic acid found in the urine might have originated in this way and that the glucuronide is less strongly bound than the parent compound.

Mycophenolic acid was apparently not extensively metabolized in the rat. Approximately 43% of the administered radioactivity was excreted in the urine (33%) and faeces (10%) 17 h after administration of a dose of 33 mg/kg. The products excreted in the urine comprised mycophenolic acid (13%), mycophenolic acid glucosiduronate (17%) and a minor uncharacterized metabolite (3%). Fig. 1 shows that the glucuronide, which was probably the phenolic *O*-derivative (II), was almost quantitatively converted into mycophenolic acid by the action of a bacterial β-glucuronidase (Ketodase); this result was confirmed by isotope dilution analysis of the urine extract before and after the enzymic hydrolysis. None of the three urinary metabolites was changed by the action of a sulphatase (Taka-diastase).

Since the [^{14}C]mycophenolic acid used was biosynthesized from [1-^{14}C]acetate, alternate carbon atoms of the biosynthetic acid might have been radioactively labelled. Metabolic breakdown of the molecule might generate radioactive one-carbon or two-carbon precursors of tissue components; hence, part of the bound tissue radioactivity

might result from incorporation, by normal biosynthetic pathways, of such precursors arising from the methyl, methoxy or side-chain groups if these were radioactively labelled. Such incorporation is likely to occur *in vivo* into tissue proteins, among other components such as lipid and carbohydrate; this is unlikely to occur significantly *in vitro* in the absence of the microsomal system or to nucleic acids *in vivo* or *in vitro* since fragments arising from radioactively labelled ethanol or acetate are not actively incorporated into rat liver nucleic acids *in vivo* or *in vitro* (Paik & Kim, 1970; Nery, 1971).

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Structural influences upon antihistamine activity; 3-amino-1-aryl-1-(2-pyridyl)propenes and related compounds

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A series of 3-amino-1-aryl-1-(2-pyridyl)propenes and related compounds have been prepared by the dehydration of corresponding tertiary alcohols, and the configurations of geometrical isomeric derivatives established from spectroscopic data. The ability of the aminopropenes to antagonize histamine-induced contractions of the guinea-pig ileum is reported. These data allow various structure-activity relations to be discussed including stereochemistry (*cis* 2-pyridyl/H geometry is superior to *cis* substituted phenyl/H but not necessarily to *cis* phenyl/H), nature of the basic function (1-pyrrolidino is markedly superior to either dimethylamino or 1-piperidino) and the importance of the vinylic hydrogen atom (sharp falls in potency follow its replacement by methyl).

The work reported was carried out to extend knowledge of structure-activity relations in 1,1-diaryl-3-aminopropenes I with antihistamine properties such as triprolidine

Ar(Ar′)C=C(H)CH_2X I   Ar(Ar′CH_2)C = CH·CH_2NRR′ II

(I, Ar = 2-pyridyl, Ar′ = *p*-Me·C_6H_4, X = 1-pyrrolidino). Data upon *cis-trans* pairs were sought in particular in view of previous reports upon the influence of geometrical configuration upon activity in isomeric 3-amino-1-aryl-1-(2-pyridyl)propenes (Adamson, Barrett & others, 1951) and 4-amino-1,2-diarylbut-2-enes II (Casy & Ison, 1970).

CHEMISTRY AND CONFIGURATION

Most of the synthetic work involved treating a Mannich base III with 2-pyridyl lithium to give a tertiary alcohol IV (Ar′ = 2-pyridyl) which was then dehydrated under acid conditions to form the aminopropenes V (Ar′ = 2-pyridyl). Isomers arose in cases where Ar ≠ Ar′ and in most of these at least one form was isolated in a pure condition whilst the composition of mixtures was known from their pmr characteristics. The proportions of isomeric alkenes formed was dependent on

ArC(=O)·CH(R)·CH_2X III   Ar·Ar′·C(OH)CH(R)·CH_2X IV   Ar(Ar′)C = C(R)·CH_2X V

(a) X = NMe_2 (b) X = 1-pyrrolidino (c) X = 1-piperidino

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their equilibration rates since the conditions employed to dehydrate the precursors IV permitted isomerization. Thus, while a 2 h exposure of IVa (Ar= Ph, Ar′ = 2-pyridyl, R = H) to hot acid gave significant amounts of the two possible isomers V, that of IVb (Ar = *p*-Me·C_6H_4, Ar′ = 2-pyridyl, R = H) yielded a single aminopropene. Kinetic control of the last reaction was achieved by reducing the heating period to 15 min when approximately equal amounts of the two isomers formed. The accelerating effect of the *p*-tolyl group upon the equilibration of isomeric alkenes has previously been noted in studies of 4-aryltetrahydropyridines (Casy, Beckett & Iorio, 1967). Isomers were separated by fractional crystallization of the hydrogen oxalate salts of the total aminoalkenes V, progress of purification being monitored by pmr spectroscopy.

The configurational assignment of isomers derived from IVa (Ar = Ph, Ar′ = 2-pyridyl, R = H) and IVb (Ar = *p*-Me·C_6H_4, Ar′ = 2-pyridyl, R = H) was initially based on differences in their ultraviolet spectra as described by Adamson, Barrett & others (1957, 1958). In both pairs, the isomeric oxalate V having an ultraviolet spectrum similar to that of 2-vinylpyridine was assigned the *trans* 2-pyridyl/CH_2N configuration (Table 1). Differences in the pmr spectra of the same isomers V were also studied, (i) to seek further information about the preferred conformations of these molecules, and (ii) to investigate the scope of pmr spectroscopy in solving stereochemical problems in this series.

Magnetic influences governing the chemical shift of the vinylic protons in isomeric pairs of type VII, deduced chiefly from data upon 2-vinylpyridine, styrene and ethylene (see Scheme 1) and assuming the additivity of shielding effects (Tobey, 1969), are as follows:

SCHEME 1

Chemical shifts (ppm from TMS, δ scale) of vinylic protons in 2-vinylpyridine and styrene (Bhacca, Johnson & Shoolery, 1962) and ethylene (Tobey, 1969) in CCl_4 or $CDCl_3$.

(1) The *cis* aryl groups—deshielding for both 2-pyridyl and benzenoid substituents but almost three fold greater in extent in the former case.

(2) The *trans* aryl groups—deshielding for 2-pyridyl, but shielding for benzenoid substituents.

Screening effects (1) and (2) will both be reduced the more the aryl group is turned out of the plane of the carbon-carbon double bond (Johnson & Bovey, 1958; Barbieux, Defay & others, 1964).

(3) The aminomethyl group—the greater the population of conformers VI with nitrogen close to the vinylic proton, the greater the deshielding of this proton. This population will depend on the extent of repulsive interactions between Ar and CH_2N substituents and is anticipated to be greater in the case of the alkene with *cis* 2-pyridyl and CH_2N groups on both steric and electronic grounds.

Because of the magnitudes of the field effects involved in (1), it follows that the vinylic signal of the *trans* isomer VII should be at lower field in the spectra of most isomeric pairs, but situations may be met (dependent on the extent of the Ar/CH_2N interaction) in which isomeric vinylic signals have very similar chemical shifts.

VI trans* cis VIII

VII

(a) X = NMe_2; Ar = Ph (b) X = 1-pyrrolidino Ar = p-Me·C_6H_4

*[2-pyridyl and aminomethyl are the configurational reference groups as proposed by Adamson & others (1957)].

Such was the case, in fact, for the isomeric pair VIIa when the bases were examined in $CDCl_3$ (see Table 1).

In spectra of the corresponding acid oxalates in D_2O, however, the *trans* signal was distinctly lower field than the *cis* vinylic resonance. Solvation of the basic centres will increase steric interactions between the 2-pyridyl and CH_2X substituents in *cis* VII [and hence decrease deshielding factor (2)] while hydrogen bonding of the type VIII will reduce the population of conformers VI, and hence reduce factor (3). Both effects will result in the vinylic signal of *cis* VII being moved to *higher* field and account for the differences in vinylic signals observed in spectra of *cis* and *trans* VIIa under these conditions. In triprolidine (*trans*-VIIb) and its *cis* isomer, the *trans* vinylic signal was lower field in the spectra of both the free bases and the oxalate salts. The result for the bases is evidence that *cis* aryl/1-pyrrolidinomethyl interactions are greater than those of aryl and dimethylaminomethyl. Comparison of data for the *trans* pair VIIa and VIIb, and the corresponding *cis* pair (Table 1) shows that the *trans* vinylic signal suffers the greater change when NMe_2 is replaced by 1-pyrrolidino. In the *trans* isomers, more pronounced Ar/CH_2N interactions will cause the vinylic signal to move downfield on two counts (reduction of benzenoid shielding and elevation of CH_2N deshielding) but will not influence the vinylic signal of *cis* isomers so much because the increase in CH_2N deshielding will be offset by the decrease in 2-pyridyl deshielding.

Table 1. *Spectral data of some 3-amino-1-1,diarylprop-1-enes.*

Compound	Chemical shift* vinylic H†	CH_2X‡	$\lambda(\epsilon)$**
trans-VIIa	6·33(6·63)	3·05(3·87)	245(7800), 283(5700)
cis-VIIa	6·33(6·37)	3·17(3·77)	252(10,000)
trans-VIIb	6·92(6·65)	3·21(4·02)	238(14,700), 283(6650)
cis-VIIb	6·27(6·45)	3·24(3·95)	244(14,100), 258(14,500)
trans-IX	6·87(6·83)	2·87(3·76)	233(14,600), 282(7500)
cis-IX	5·98(6·03)	3·42(3·93)	spectrum of pure isomer not available

* ppm(δ), base in $CDCl_3$ (TMS standard); values in parenthesis refer to acid oxalates in D_2O (DSS standard).

† triplet, $J\sim7$Hz

‡ doublet, $J\sim7$Hz

** Oxalate in ethanol. λ is wave length in nm; extinction coefficient ε shown in parenthesis. Adamson & others (1957, 1958) report; *trans*-VIIa, 238(20 900) 280(10 700; *cis*-VIIa, 247(22 400) oxalates in chloroform–ethanol; *trans*-VIIb, 233(16 200) 283(8200); *cis*-VIIb, 233(13 600) 260(13 800), oxalates in ethanol.

Shielding considerations (1) and (2) also apply to the CH_2X protons. In the pairs VIIa and VIIb, examined as bases, the *cis* signals had the lower field position as expected from the proximity of CH_2X to 2-pyridyl. In spectra of the oxalates in D_2O, however, the *trans* signals were lower field. This result is again attributed to enhanced steric interactions between 2-pyridyl and CH_2X when the basic centres of *cis* isomers are solvated; in consequence, deshielding due to factor (1) is reduced to a level where it is surpassed by deshielding factors (1) and (2) operating in the *trans* isomers. The larger steric demands of 1-pyrrolidinomethyl over CH_2NMe_2 are again revealed by the smaller differences in CH_2X chemical shifts in the spectra of *cis* and *trans* VIIb as compared with those of VIIa. Differences in ultraviolet and pmr characteristics similarly allowed the stereochemical characterization of the 1-*o*-tolyl analogues IX. In this pair, the CH_2X signal was at lower field in the spectra of both free base and oxalate of the *cis* member (Table 1); this result is attributed to the very large interaction between *o*-tolyl and CH_2X substituents in the *trans* isomer which forces the aromatic group well out of the plane of the carbon-carbon double bond whereby the CH_2X protons are shielded rather than deshielded by the *o*-tolyl group.

Reaction of the Mannich bases III a-c (Ar = Ph, R = Me) with 2-pyridyl lithium was essentially stereospecific since the pmr spectra of the products IV a-c (Ar = Ph, Ar′ = 2-pyridyl, R = Me) displayed no duplication of signals as would be typical of a diastereoisomeric mixture. Dehydration of the alcohols IV a-c (Ar = Ph, Ar′ = 2-pyridyl, R = Me) gave an approximately equal mixture of *cis-trans* alkenes X in each case; fractional crystallization of corresponding oxalates yielded one isomerically pure form of Xa and mixtures of Xb and Xc of known composition. Configurational assignments were based on the *cis* 2-pyridyl/CH_2X isomers having the lower field CH_2X and higher field =C·Me resonances. Differences in the latter signals were small and were only revealed in the case of the 3-piperidino analogue Xc when the spectrum of a mixture was recorded in benzene, the result being a further example of the ASIS effect (Laszlo, 1967). Several non-isomeric 1,1-diaryl-3-aminopropenes V which did not contain the 2-pyridyl moiety were also prepared and tested (details in Table 2). The aminoalkene Vb (Ar = Ph, Ar′ = *p*-Me·C_6H_4, R = H) was isolated as a 50:50 mixture of geometrical isomers, as shown by the duplication of the aryl Me and CH_2N signals in the pmr spectrum of the product.

IX X

(a) X = NMe_2
(b) X = 1-pyrrolidino
(c) X = 1-piperidino

PHARMACOLOGICAL RESULTS AND DISCUSSION

The antihistamine potencies of the 3-amino-1,1-diarylprop-1-enes and related compounds, as measured by their ability to antagonize the histamine-induced con-

Table 2. *Inhibition of histamine-induced contractions of isolated guinea-pig ileum by some 3-amino-1-aryl-1-(2-pyridyl)propenes and related compounds**.

Compound No.	Configuration†	Structure Text formula	Basic substituent	Concn µg/ml	% inhibition at time (in min) :3	:6	:9	:12	:15	:18	:21	:24
1	*trans*	VIIa	NMe_2	0·10	32	30	23	0	—	—	—	—
2	*cis*	VIIa	NMe_2	0·10	40	32	0	—	—	—	—	—
	Mepyramine			0·001	49	36	27	25	19	15	—	—
3	*trans*	VIIb	1-pyrrolidino	1·0	100	100	72	83	63	—	—	—
				0·01	59	45	24	14	0	—	—	—
4	*cis*	VIIb	1-pyrrolidino	1·0	61	54	25	29	25	21	18	11
	Mepyramine			0·001	50	32	22	15	10	9	4	—
5	*trans*	IX	NMe_2	0·10	100	85	65	50	40	25	15	15
				0·01	63	31	13	0	0	—	—	—
6	*cis*(50) : *trans*(50)	IX	NMe_2	1·0	100	90	56	27	27	8	0	—
	Mepyramine			0·001	94	77	47	31	15	0	—	—
7	*cis*	Xa	NMe_2	0·10	33	24	14	5	—	—	—	—
8	*cis*(30) : *trans*(70)	Xa	NMe_2	0·10	19	19	10	19	19	10	10	—
	Mepyramine			0·001	49	36	27	25	19	15	—	—
9	*cis*(85) : *trans*(15)	Xb	1-pyrrolidino	1·0	48	8	4	—	—	—	—	—
10	*cis*(50) : *trans*(50)	Xc	1-piperidino	1·0	32	0	—	—	—	—	—	—
	Mepyramine			0·001	100	96	76	57	38	27	13	13
11	Va(Ar = Ar′ = Ph,R = H)		NMe_2	0·10	60	40	15	0	—	—	—	—
12	Va(Ar = Ar′ = Ph,R = Me)		NMe_2	0·10	0	—	—	—	—	—		—
13	3-Dimethylamino-3-methyl-1,1-diphenylpropene		NMe_2	0·10	32	25	17	—	—	—	—	—
	Mepyramine			0·001	49	36	27	25	19	15	—	—
14	Vb(Ar = Ar′ = Ph,R = H)		1-pyrrolidino	0·01	100	90	47	47	42	32	26	26
				0·001	88	50	44	19	0	0	—	—
15	Vb(Ar = Ar′ = *p*-Me·C_6H_4, R = H)		1-pyrrolidino	0·01	48	33	29	19	24	14	14	—
	Mepyramine			0·001	91	91	86	77	73	64	64	60
16	*cis*(50) : *trans*(50)Vb(Ar = Ph, Ar′ = *p*-Me·C_6H_4,R = H)		1-pyrrolidino	1·0	100	100	100	100	—	—	—	—
				0·01	39	21	11	14	7	—	—	—
	Mepyramine			0·001	50	32	22	15	10	9	4	—
17	*trans*(H/Ph)-1-*p*-Chlorophenyl-2-phenyl-4-(1-pyrrolidino)but-2-ene			0·10	82	75	46	57	45	45	38	—
				0·001	24	19	14	9	0	—	—	—
18	*trans*(Ar/Ar′)-1-*p*-Chlorophenyl-2-phenyl-4-(1-pyrrolidino)but-1-ene‡			0·01	69	65	62	53	56	25	16	37
19	*cis*(Ar/Ar′)-1-*p*-Chlorophenyl-2-phenyl-4-(1-pyrrolidino)but-1-ene			0·01	45	41	28	17	0			
	Mepyramine			0·001	49	36	27	19	15			

* tested as hydrogen oxalates or hydrohalides
† reference groups are 2-pyridyl and aminomethyl unless otherwise stated
‡ pA_2 7·4

traction of the guinea-pig ileum are given in Table 2. Thanks are due to Dr. R. T. Brittain of Allen and Hanburys, Ware, Herts., for arranging these tests. The compounds were tested in groups on separate occasions and corresponding responses of the tissue to the standard drug mepyramine are included in the Table. Apart from cases where pA_2 values were measured, these data only allow semi-quantitative activity comparisons. Nevertheless certain trends in structure-activity relations are discernible, as discussed below.

(1) Comparison of data for the pairs No. 1/3 and No. 11/14 shows that a pronounced enhancement of potency follows replacement of dimethylamino by 1-pyrolidino in 3-amino-1-aryl-1-(2-pyridyl)propenes. A similar observation was made for a series of antihistaminic 3-amino-1,1-diphenylpropenes (White, Green & Hudson, 1951). These results are somewhat surprising in view of the similar basic properties and gross dimensions of the two amino functions (see later). Related 1,2-diaryl-4-(1-pyrrolidino)butenes also possess significant antihistamine potencies and there appears to be a diminished stereospecific dependence upon activity amongst 4-(1-pyrrolidino)butenes compared with that found for 4-dimethylamino and 4-piperidino analogues (Casy & Ison, 1970). Thus both the *cis* and *trans* but-1-enes (Nos. 18 and 19) are moderately potent while the *trans* but-2-ene (No. 17) has a pA_2 approaching 8. Pyrrobutamine,

the *cis* analogue of the last compound, which is in clinical use is confirmed as a very potent antihistamine agent but its pA_2 value could not be determined because it had a non-competitive mechanism of action.

(2) *Stereospecificity*. Adamson & others (1951) have made the general statement that for drugs of the triprolidine class I "high and specific antihistamine activity was shown only by isomers having the α-pyridylethylene type of structure (i.e. *trans* 2-pyridyl/CH_2N isomers), the other isomer of each pair invariably being considerably less active in this respect". Particular examples were limited to the isomeric pair I (Ar = 2-pyridyl, Ar′ = *p*-Cl·C_6H_4, X = 1-pyrrolidino), the *trans* (2-pyridyl/CH_2N) member being reported as highly potent and 80 times as active as the corresponding *cis* isomer. The data of Table 2 now provides information on a further two pairs of isomers of this class. Triprolidine itself (No. 3) is highly potent (pA_2 9·0) and is more active than the corresponding *cis* isomer (No. 4); the potency difference appears to be of a lower order, however, than that anticipated from the 80 fold difference reported for 1-(2-pyridyl)-1-*p*-chlorophenyl analogues. Both *cis* and *trans* 1-(2-pyridyl)-1-phenyl derivatives (Nos. 1 and 2) have feeble antihistamine activities of short duration and there is no noticeable difference in their potencies. These results raise the question of the general superiority (from a pharmacological point of view) of 2-pyridyl over phenyl and substituted phenyl as the aryl group *trans* to aminomethyl in antihistamines of structure I. This appears to be true when the choice is between 2-pyridyl and *p*-chlorophenyl or *p*-tolyl but is less certain in the case of phenyl itself in view of results on 1,1-diphenyl-3-pyrrolidinoprop-1-ene (No. 14). This compound, although short acting has a high potency with a pA_2 (after a 2 min contact time) determined as well above the value 9 established for triprolidine (*cf.* White & others, 1951). The significant potency of the *cis-trans* mixture No. 16 (pA_2 8·5) further demonstrates that non-yyridyl containing analogues of I retain pronounced antihistamine properties while the lower activity of No. 15 compared with No. 14 shows that phenyl is preferred to *p*-tolyl as the aromatic group *trans* to CH_2N in I. A clear decision regarding the relative efficacies of phenyl and 2-pyridyl in this respect must await the pharmacological evaluation of *cis* and *trans* 1-phenyl-1-(2-pyridyl)-3-pyrrolidinoprop-1-ene.

(3) Results from compounds Nos. 7–10 establish the importance of having a vinylic hydrogen atom *cis* to an aromatic group in antihistamines based on I. Since the aryl substituents are phenyl and 2-pyridyl, the activities of these compounds should not be greatly dependent upon configuration (see 2 above) and the uniformly low orders of potency observed emphasizes the detrimental effect of an increase in the bulk of the substituent *cis* to Ar in I. The same trend is apparent in Nos. 11 and 12. A possible explanation of this result may be the less favoured nature of a coplanar Ar·C = C conformation in 2-methyl analogues of I as a result of *cis* Ar/Me interactions. An essentially coplanar Ar·C = C·C·N arrangement has previously been advanced as an important requirement for antihistamine activity in 1,1-diarylprop-1-enes and 1,2-diarylbut-2-enes (Casy & Ison, 1970). Another result in support of this view and appropriate to this section is the reduced activity of the 2-*o*-tolylbut-2-

-Me
C=C
H
$PhCH_2$
CH_2NMe_2

XI

ene XI (in which a coplanar Ar·C = C conformation is likewise unfavoured) as compared with the 2-phenyl analogue (Ison, 1970).

(4) In contrast with the 2-methyl analogues of I, compound No. 5 is an example in which an increased divergence of the aryl group Ar′ from the double bond plane is achieved as is evident from models and physical evidence already discussed. Although direct comparison of Nos. 5/6 and the unsubstituted phenyl analogues Nos. 1/2 is not possible because the two sets of isomers were tested on separate occasions, it is clear that (i) both the *trans* and *cis/trans* mixture (Nos. 5 and 6) are significantly active, (ii) the *trans* derivative (No. 5) is more potent than the *cis* isomer (No. 6), and (iii) the *trans o*-tolyl derivative (No. 5) is more potent than the phenyl congener (No. 1). These results suggest that factors which increase the deviation of the Ar′ and C = C planes in antihistamines of structure I have an advantageous effect upon potency either in terms of association of Ar′ at the receptor or because of a concomitant increase in the population of planar Ar·C = C conformers. In crowded molecules there is evidence that the steric requirements of pyrrolidino are significantly greater than those of dimethylamino, piperidino and morpholino (Munk & others, 1968) (see also pmr discussion). Hence the potency raising power of the pyrrolidino group in antihistamines of structure I may lie in its influence upon the orientation of the adjacent aromatic substituent (Ar′).

EXPERIMENTAL CHEMISTRY

Tertiary alcohols (IV). The following *2-pyridyl carbinols* were prepared by treatment of the appropriate Mannich ketones (III) (all previously described) with 2-pyridyl lithium at −50° under N_2 according to the method of Adamson & Billinghurst (1950) (yields in parenthesis): IVa (Ar = Ph, Ar′ = 2-pyridyl, R = H), (53%), m.p. 97–99° from ethanol, reported m.p. 99–100° (Adamson & Billinghurst, 1950); IVb (Ar = *p*·Me·C_6H_4, Ar′ = 2-pyridyl, R = H), (28%), m.p. 116–117° from ethanol, reported m.p. 119–120° (Adamson & others, 1958); IVa (Ar = *o*-Me·C_6H_4, Ar′ = 2-pyridyl, R = H), (11%), m.p. 96° from ethanol (Found: C, 75·8; H, 8·4. $C_{17}H_{22}N_2O$ requires: C, 75·5; H, 8·2%); IVa (Ar = Ph, Ar′ = 2-pyridyl, R = Me), (49%), m.p. 74–75° from ethanol (Found: C, 75·6; H, 8·0; N, 10·1. $C_{17}H_{22}N_2O$ requires: C, 75·5; H, 8·2%;) IVb (Ar = Ph, Ar′ = 2-pyridyl, R = Me), (9%), m.p. 112° from hexane, reported m.p. 111° (Adamson & others, 1958); IVc (Ar = Ph, Ar′ = 2-pyridyl, R = Me), (18%), m.p. 125–126° from hexane reported m.p., 134° (Adamson, & others, 1958) (Found: C, 77·4; H, 8·4: N, 9·3. $C_{20}H_{26}N_2O$ requires: C, 77·4; H, 8·4; N, 9·0%).

The *diphenyl carbinols* IVa and IVb (Ar = Ar′ = Ph, R = H) (Adamson, 1949), IVa (Ar = Ar′ = Ph, R = Me) and 3-dimethylamino-1,2-diphenyl-1- methylpropan-1-ol (Kjaer & Peterson, 1951) were all prepared using phenyl lithium as the aryl organometallic reagent.

The *p-tolyl carbinols* IVb (Ar = Ph, Ar′ = *p*-Me·C_6H_4, R = H) and IVb (Ar = Ar′ = *p*-Me·C_6H_4, R = H) were prepared by treatment of the aminoketone IIIb (Ar = *p*-Me·C_6H_4, R = H) with phenyl lithium in toluene and *p*-tolyl lithium in ether respectively, followed by the normal workup. Thus were obtained IVb (Ar = Ph, Ar′ = *p*-Me·C_6H_4, R = H) *hydrochloride*, (16%), m.p. 167–168° from ethanol ether (Found: C, 72·2; H, 8·05. $C_{20}H_{25}NO{\cdot}HCl$ requires- C, 72·4; H, 7·9%) and IVb (Ar = Ar′ = *p*-Me·C_6H_4, R = H) *free base*, (60%), m.p. 131–132° from ethanol (Found: C, 81·6; H, 8·6. $C_{21}H_{27}NO$ requires: C, 81·5; H, 8·8%).

The 3-amino-1,1-diarylpropenes (V) were prepared by acid-catalysed dehydration of the appropriate tertiary alcohols. In the case of derivatives containing the 2-pyridyl group, the dehydrating medium used was 85% sulphuric acid as previously reported (Adamson & Billinghurst, 1950) with 2 h reaction times at 100–110° being used except in the case of IVb (Ar = *p*-Me·C_6H_4, Ar′ = 2-pyridyl, R = H) (see main text). The liberated free base mixtures were acidified with ethanolic oxalic acid and fractional crystallization produced either pure salts or isomer mixtures (pmr evidence). The remaining carbinols were dehydrated using an acetic acid and concentrated hydrochloric acid mixture according to the method of Casy, Myers & Pocha (1966). Thus were obtained the following compounds and isomeric mixtures (all recrystallized from ethanol–ether): trans-VIIa *hydrogen oxalate*, m.p. 176°, reported m.p. 179° (Adamson & others, 1957) (Found: C, 65·6; H, 6·3; N, 8·4. $C_{18}H_{20}N_2O_4$ requires: C, 65·8; H, 6·1; N, 8·5%); cis-VIIa *hydrogen oxalate*, m.p. 178–179°, reported m.p. 180–181° (Adamson & others, 1957) (Found: C, 65·8; H, 6·0; N, 8·7%); trans VIIb *hydrogen oxalate*, m.p. 171°, reported m.p. 173–174° (Adamson & others, 1958); cis VIIb *hydrogen oxalate*, m.p. 147–148°, reported m.p. 149–150° (Adamson, & others, 1958); trans IX *hydrogen oxalate*, m.p. 160° (Found: C, 66·6; H, 6·5; N, 8·1. $C_{19}H_{22}N_2O_4$ requires: C, 66·65; H, 6·5; N, 8·2%).

The pure oxalate of *cis* IX could not be isolated but its pmr characteristics were noted from various mixed oxalate crops (see Table 1). Liberation of the bases from these samples and reacidification with ethanolic hydrogen bromide led to the isolation of a 50:50 mixture (pmr integral data) of the cis *and* trans *IX dihydrobromides*, m.p. 213–214° (d.) (Found: C, 49·1; H, 5·5. $C_{17}H_{20}N_2$·2HBr requires: C, 49·3; H, 5·4%) which was used in pharmacological testing (compound 6, see Table 2).

cis Xa *Hydrogen oxalate*, m.p. 184–185° (Found: C, 66·8; H, 6·6; N, 8·1. $C_{19}H_{22}N_2O_4$ requires: C, 66·65; H, 6·5; N, 8·2%). Pmr characteristics in ppm (δ), base in $CDCl_3$ (TMS), values in parenthesis refer to hydrogen oxalates in D_2O (DSS): CH_2N, 2·97 (3·87) (singlet), = C·Me, 1·85 (1·80) (singlet). Also isolated was a 70:30 mixture (pmr integral data) of the trans *and* cis Xa *hydrogen oxalates*, m.p. 158° (compound 8, Table 2), from which the following pmr characteristics were obtained for the *trans* isomer CH_2N, 2·47 (3·72) (singlet), = C·Me 1·90 (1·84) (singlet).

No pure isomers could be isolated from either of the elimination products derived from the IVb or IVc (Ar = Ph, Ar′ = 2-pyridyl, R = Me) carbinols. However, the following enriched mixtures were obtained: An 85:15 mixture of the cis *and* trans Xb *hydrogen oxalates*, m.p. 154–155° (compound 9, Table 2), (Found: C, 68·2; H, 6·4. $C_{21}H_{24}N_2O_4$ requires: C, 68·45; H, 6·6%). Pmr characteristics in ppm (δ), bases in $CDCl_3$ (TMS), values in parenthesis refer to hydrogen oxalates in D_2O (DSS): *cis* isomer, CH_2N, 3·18 (3·97) (singlet), = C·Me, 1·92 (1·89) (singlet); *trans* isomer, CH_2N, 2·94 (3·83) (singlet), = C·Me, 1·94 (1·89) (singlet).

A 50:50 mixture of the cis *and* trans Xc *oxalates*, m.p. 159–160° (compound 10, Table 2), (Found: C, 69·25; H, 6·8. $C_{22}H_{26}N_2O_4$ requires: C, 69·1; H, 6·85%). Pmr characteristics (specified above) of the mixture: CH_2N, 2·95 (3·70 and 3·87) (singlets), = C·Me, 1·87 (1·88) (singlets). Separation of the superimposed isomeric signals (see above) was achieved by running a pmr spectrum of the free base mixture in benzene (TMS) which revealed the following resonance absorptions: CH_2N, 3·03 and 3·13, = C·Me, 2·02 and 2·12 ppm (δ).

The following aminopropenes were prepared from the appropriate tertiary alcohols using an acetic acid and concentrated hydrochloric acid dehydration medium (see

earlier): Va (Ar = Ar′ = Ph, R = H) hydrochloride, m.p. 168°, reported m.p. 168–170° (Adamson, 1949); Va (Ar = Ar′ = Ph, R = Me) hydrochloride, m.p. 190°, reported m.p. 191° (Kazaryan & Nazarov, 1957); Vb (Ar = Ar′ = Ph, R = H) hydrochloride, m.p. 163°, reported m.p. 165–167° (Adamson, 1949); 1,1-diphenyl-3-methyl-3-dimethylaminoprop-1-ene (cmp. 13, Table 2) hydrochloride, m.p. 161°, reported 162–163° (Casy, Beckett & Armstrong, 1961); 50:50 mixture of *cis* and *trans* Vb (Ar = Ph, Ar′ = *p*-Me·C_6H_4, R = H) *hydrochlorides* (cmp. 16, Table 2), m.p. 143–144° from ethanol–ether (Found: C, 76·5; H, 7·8. $C_{20}H_{23}N·HCl$ requires: C, 76·3; H, 7·7%), pmr characteristics in ppm (δ), bases in $CDCl_3$ (TMS), values in parenthesis refer to hydrochlorides in $CDCl_3$ (TMS): = C·H, 6·22 (6·45) (superimpose triplets, *J*7), CH_2N, 3·15 and 3·18 (hydrochloride masked by pyrrolidino bands) Ar·Me, 2·30 and 2·36 (2·32 and 2·38) (singlets); Vb (Ar = Ar′ = *p*-Me·C_6H_4, R = H) *hydrochloride*, m.p. 217° from ethanol–ether (Found: C, 76·8; H, 8·0. $C_{21}H_{25}N·HCl$ requires: C, 76·9; H, 8·0%) pmr characteristics (specified above): = C·H, 6·23 (6·46) (triplet), *J*7), CH_2N, 3·27 (3·75) (doublet, *J*7), Ar·Me, 2·32 and 2·38 (2·33 and 2·40) (singlets).

The pyrrobutamine isomers (compounds 17, 18 and 19, Table 2) and pyrrobutamine itself have been previously described (Casy & Ison, 1970).

Pmr spectra were recorded on a Varian A-60D instrument and ultraviolet spectra on a Beckmann DK-2 recording spectrophotometer.

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The effects of substituting tetrazole for carboxyl in two series of anti-inflammatory phenoxyacetic acids

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Series of *o*-phenylcarbamoyl- and *o*-benzamido-phenoxymethyl tetrazoles and *o*-phenylcarbamoylphenoxyacetic acids have been synthesized. Anti-inflammatory activity was measured by the phenyl benzoquinone writhing test in mice and the rat foot carrageenan oedema test. Potency in the two *o*-benzamido substituted series could not be related with structure in a satisfactory manner. Introduction of substituents into the benzene rings of the *o*-phenylcarbamoyl substitued series led to complex changes. When the phenoxy ring was unsubstituted, introduction of *meta*- and *para*-substituents possessing high +ve π constants into the *o*-phenylcarbamoyl ring led to increased potency, and each tetrazole was appreciably more potent than the corresponding acid. When the *o*-phenylcarbamoyl ring was unsubstituted *meta*- and *para*-substituents with high +ve π constants introduced into the phenoxy ring caused increases in potency in the acid series but not in the tetrazole series, and each acid was more potent than the corresponding tetrazole. The two tetrazoles found to be the most active in the mouse writhing test 5-[2-(3,4-dichlorophenylcarbamoyl)phenoxymethyl]tetrazole (compound 12T, SNR.2337) and 5-[4-chloro-2-(3-trifluoromethylphenylcarbamoyl)phenoxymethyl]tetrazole (compound 22T, SNR.2420) were selected for study in a series of other anti-inflammatory tests.

The preparation and anti-inflammatory activity of a series of phenyl- and phenoxyalkanoic acids have been described previously (Drain, Daly & others, 1970). In addition to modification of the acidic side chain other structural variations were considered which might lead to compounds with higher activity.

The tetrazole group has an acidic hydrogen which is known to compare with the carboxyl group in pK_a (Mihina & Herbst, 1950; McManus & Herbst, 1959), and as a result several workers have examined tetrazole analogues of physiologically active carboxylic acids in a variety of suitable test systems. Straaten, Solinger & others (1958) found that 5-(4-aminophenyl)tetrazole, the analogue of *p*-aminobenzoic acid, was inactive against *Staphylococcus aureus* and other micro-organisms and the tetrazole analogues of *p*-aminosalicylic acid and isonicotonic acid were inactive against *Mycobacterium tuberculosis.* However, the tetrazole analogue of nicotinic acid assayed as a growth factor substitute for *Lactobacillus arabinosus* showed some activity. McManus & Herbst (1959) prepared a series of tetrazole analogues of amino-acids which were tested (Zygmunt, 1962) as inhibitors of bacterial growth. The compounds were either inactive or very weak growth inhibitors. More recently, Juby, Hudyma & Brown (1968) synthesized a series of 5-(2-anilinophenyl)tetrazoles as analogues of flufenamic acid. They found that the anti-inflammatory activity of each tetrazole was very similar to its corresponding acid, indicating that the replacement of the carboxyl group by tetrazole could lead to active anti-inflammatory agents.

Table 1. *2-Benzamidophenoxyacetic acids and 5-(2-benzamidophenoxymethyl)tetrazoles.*

Cpd. No.	Subt. Ring P	Subt. Ring Q	Method No.	Yield (%)	m.p.[1] °C	Recrystn. solvent	Formula[2]	LD50 mg/kg (oral)	P.B.Q. test (oral)* (mg/kg)	P.B.Q. test (oral)* (μmol/kg)	RFT† (oral)[3]
1A	H	H	1A[4]					>1000	140	520	−
1T	H	H	2	64	180–1	Me_2CO-H_2O	$C_{15}H_{13}N_5O_2$	>1000	53	180	+
2A	H	4-Me	1B[4]					>1000	40	140	−
2T	H	4-Me	2	72	194–5	$DMF-H_2O$	$C_{16}H_{15}N_5O_2$	>1000	80	260	−
3A	H	3,4-Cl_2	1B					>1000	31	91	+
3T	H	3,4-Cl_2	2	90	203–4	Me_2CO-H_2O	$C_{15}H_{11}Cl_2N_5O_2$	>1000	21	58	+
4A	4-Cl	4-Cl	1B					700	150	440	±
4T	4-Cl	4-Cl	2	80	231–2	$DMF-H_2O$	$C_{15}H_{11}Cl_2N_5O_2$	>1000	22	61	+
5A	4-Cl	3,4-Cl_2	1B					>1000	29	78	+
5T	4-Cl	3,4-Cl_2	2	75	223–4	$DMF-H_2O$	$C_{15}H_{10}Cl_3N_5O_2$	>1000	17	43	+
6A	4-Me	4-Cl	1B					750	11	35	−
6T	4-Me	4-Cl	2	75	184–5	$DMF-H_2O$	$C_{16}H_{14}ClN_5O_2$	>1000	30	87	−
7A	4-Me	3,4-Cl_2	1B					1000	44	120	+
7T	4-Me	3,4-Cl_2	2	75	202–3	$DMF-H_2O$	$C_{16}H_{13}Cl_2N_5O_2$	>1000	42	110	+
8A	4-Me	3-CF_3	1B					750	27	77	−
8T	4-Me	3-CF_3	2	78	186–7	Me_2CO-H_2O	$C_{17}H_{14}F_3N_5O_2$	>1000	10	27	+
9A	5-OMe	4-Cl	1B					>1000	67	200	−
9T	5-OMe	4-Cl	2	90	237–8	$DMF-H_2O$	$C_{16}H_{14}ClN_5O_3$	>1000	90	250	−
10A	5-OMe	3,4-Cl_2	1B					1000	77	210	−
10T	5-OMe	3,4-Cl_2	2	90	219–20	$DMF-H_2O$	$C_{16}H_{13}Cl_2N_5O_3$	>1000	90	230	−

* 50% reduction in writhing rate dose.
† Rat foot test (activity at 50 mg/kg).
[1] Melting points are uncorrected.
[2] All compounds were analysed for C,H,N and analytical results obtained for these elements were within ±0·4% of the theoretical values.
[3] *P* values for *t*-tests on rat foot oedema activity are as follows: + = $P < 0·05$, ± = $P < 0·1$ to $> 0·05$, − = $P > 0·1$.
[4] Acids prepared by methods 1A and 1B according to Drain & others (1970).

The 2-benzamidophenoxyacetic acids have previously been described in detail (Drain & others, 1970) and are included here for purposes of comparison. In an extension of this work the 2-phenylcarbamoylphenoxyacetic acids have been prepared together with the tetrazole analogues of both acid series.

The methods for assessing anti-inflammatory activity were the PBQ-induced mouse writhing test and the carrageenan rat foot oedema test.

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The compounds of Tables 1 and 2 were prepared by several methods which are illustrated in the following examples.

Melting points were recorded on a Gallenkamp manual melting point apparatus.

Methods 1A and 1B

For details of these methods of preparation see footnote (4) under Table 1.

Method 2

5-[2-(3,4-*Dichlorobenzamido*)*phenoxymethyl*]*tetrazole* (*cpd no.* 3*T*). 2-(3,4-*Dichlorobenzamido*)*phenoxyacetonitrile*. To a solution of 2-(3,4-dichlorobenzamido)phenol (5·64 g; 0·02, mol) and chloroacetonitrile (1·89 g; 0·025 mol) in Me_2CO (40 ml) was

Table 2. *2-Phenylcarbamoylphenoxyacetic acids and 5-(2-phenylcarbamoylphenoxymethyl)tetrazoles.*

Cpd. No.	Subt. Ring P	Subt. Ring Q	Method No.	Yield (%)	m.p.[1] °C	Recrystn. solvent	Formula[2]	LD50 mg/kg (oral)	P.B.Q. test (oral)* (mg/kg)	P.B.Q. test (oral)* (μmol/kg)	RFT† (oral)[3]
11A	H	H	4	89	160–1	Me_2CO-H_2O	$C_{15}H_{13}NO_4$[4]	> 1000	60	220	–
11T	H	H	2	80	167–8	$DMF-H_2O$	$C_{15}H_{13}N_5O_2$	> 1000	15	51	+
12A	H	3,4-Cl_2	3	87	261–2	AcOH	$C_{15}H_{11}Cl_2NO_4$	> 1000	10	29	+
12T	H	3,4-Cl_2	2	85	199–200	AcOH	$C_{15}H_{11}Cl_2N_5O_2$	800	2·1	5·8	+
13A	H	3,5-Cl_2	4	65	218–9	$DMF-H_2O$	$C_{15}H_{11}Cl_2NO_4$	500	12	35	+
13T	H	3,5-Cl_2	2	75	220–1	Me_2CO-H_2O	$C_{15}H_{11}Cl_2N_5O_2$	> 1000	2·7	7·4	+
14A	H	3-CF_3	3	46	215–7	n-Bu_2O	$C_{16}H_{12}F_3NO_4$	1000	19	56	+
14T	H	3-CF_3	2	70	182–3	Me_2CO-H_2O	$C_{16}H_{12}F_3N_5O_2$	> 1000	15	41	+
15A	H	4-OMe	4	80	176–7	Me_2CO-H_2O	$C_{16}H_{15}NO_5$	> 1000	100	330	–
15T	H	4-OMe	2	80	172–3	Me_2CO-H_2O	$C_{16}H_{15}N_5O_3$	600	13	40	–
16A	4-Cl	H	3	90	202–3	Me_2CO-H_2O	$C_{15}H_{12}ClNO_4$	> 1000	7·5	25	+
16T	4-Cl	H	2	70	188–9	Me_2CO-H_2O	$C_{15}H_{12}ClN_5O_2$	1000	23	70	–
17A	4-Br	H	4	60	206–7	n-BuOH	$C_{15}H_{12}BrNO_4$	> 1000	3·0	8·6	+
17T	4-Br	H	2	77	176–7	$AcOH-H_2O$	$C_{15}H_{12}BrN_5O_2$	> 1000	20	54	–
18A	4-Me	H	4	86	199–200	Me_2CO-H_2O	$C_{16}H_{15}NO_4$	> 1000	4·6	16	+
18T	4-Me	H	2	85	170–1	Me_2CO-H_2O	$C_{16}H_{15}N_5O_2$	750	36	120	–
19A	4-CMe_3	H	4	70	204–5	Me_2CO-H_2O	$C_{19}H_{21}NO_4$	500	6·0	18	+
19T	4-CMe_3	H	2	90	202–3	Me_2CO-H_2O	$C_{19}H_{21}N_5O_2$	> 1000	40	110	–
20A	4-Cl	3,4-Cl_2	3	25	260–1	DMF-n-Bu_2O	$C_{15}H_{10}Cl_3NO_4$	> 1000	11	29	+
20T	4-Cl	3,4-Cl_2	2	50	230–1	$DMF-H_2O$	$C_{15}H_{10}Cl_3N_5O_2$	> 1000	3·4	8·5	+
21A	4-Cl	4-Me	3	50	217–8	n-Bu_2O	$C_{16}H_{14}ClNO_4$	750	22	69	±
21T	4-Cl	4-Me	2	80	191–2	$DMF-H_2O$	$C_{16}H_{14}ClN_5O_2$	750	30	87	+
22A	4-Cl	3-CF_3	4	75	214–5	Me_2CO-H_2O	$C_{16}H_{11}ClF_3NO_4$	500	2·0	5·4	+
22T	4-Cl	3-CF_3	2	75	212–3	Me_2CO-H_2O	$C_{16}H_{11}ClF_3N_5O_2$	500	1·4	3·5	+
23A	4-Br	3-CF_3	4	64	189–90	i-PrOH	$C_{16}H_{11}BrF_3NO_4$	> 1000	64	150	–
23T	4-Br	3-CF_3	2	73	221–2	AcOH	$C_{16}H_{11}BrF_3N_5O_2$	375	24	54	–
24A	4-Me	4-Cl	4	55	236–7	Me_2CO-H_2O	$C_{16}H_{14}ClNO_4$	> 1000	30	94	–
24T	4-Me	4-Cl	2	62	192–3	Me_2CO-H_2O	$C_{16}H_{14}ClN_5O_2$	> 1000	14	41	+
25A	4-Me	3,4-Cl_2	3	49	252–3	DMF-n-Bu_2O	$C_{16}H_{13}Cl_2NO_4$	> 1000	14	40	+
25T	4-Me	3,4-Cl_2	2	70	212–3	Me_2CO-H_2O	$C_{16}H_{13}Cl_2N_5O_2$	> 1000	42	110	+
26A	4-Me	3-CF_3	3	58	225–6	n-Bu_2O	$C_{17}H_{14}F_3NO_4$	1000	13	37	±
26T	4-Me	3-CF_3	2	80	188–9	$DMF-H_2O$	$C_{17}H_{14}F_3N_5O_2$	1000	14	37	+
27A	4-Et	3,4-Cl_2	4	75	245–7	Me_2CO-H_2O	$C_{17}H_{15}Cl_2NO_4$	375	48	130	+
27T	4-Et	3,4-Cl_2	2	85	212–3	$DMF-H_2O$	$C_{17}H_{15}Cl_2N_5O_2$	> 1000	30	77	–

* 50% reduction in writhing rate dose.
† Rat foot test (activity at 50 mg/kg).
[1] Melting points are uncorrected.
[2] All compounds were analysed for C,H,N and analytical results obtained for these elements were within ±0·4% of the theoretical values.
[3] *P* values for *t*-tests on rat foot oedema activity are as follows: + = $P < 0·05$, ± = P = <0·1 to >0·05, – = $P > 0·1$.
[4] Cohn (1899).

added anhydrous K_2CO_3 (2·27 g; 0·02 mol) and the mixture was boiled under reflux with stirring for 8 h. The cold mixture was poured into aqueous 0·5N NaOH (100 ml) and the precipitate was filtered, washed (H_2O) and dried to afford 6·25 g (97%) of product, m.p. 142–3°. Recrystallization from $Me_2CO–H_2O$ gave the pure product as colourless needles, m.p. 143–4°. Analysis: ($C_{15}H_{10}Cl_2N_2O_2$) C,H,N.

5-[2-(3,4-*Dichlorobenzamido*)*phenoxymethyl*]*tetrazole.* To a solution of 2-(3,4-dichlorobenzamido)phenoxyacetonitrile (4·01 g; 0·0125 mol) in DMF (30 ml) was added NH_4Cl (0·70 g; 0·0131 mol) and NaN_3 (0·85 g; 0·0131 mol), and the mixture was heated on a steam bath with stirring for 18 h. The solvent was removed *in vacuo* and the resulting oil was dissolved in 1·5N NH_4OH solution (100 ml) and extracted

with EtOAc. The ammoniacal solution was acidified to pH 2 (HCl) and the precipitate was filtered, washed (H_2O) and dried to give 4·48 g (98%) of cpd no. 3T, m.p. 194–5°. Recrystallization from Me_2CO–H_2O afforded 3·88 g (85%) of the pure product as colourless needles, m.p. 203–4°. Analysis: ($C_{15}H_{11}Cl_2N_5O_2$) C,H,N.

Method 3

4-*Methyl*-2-(3-*trifluoromethylphenylcarbamoyl*)*phenoxyacetic acid* (*cpd no.* 26*A*). 4-*Methyl*-2-(3-*trifluoromethylphenylcarbamoyl*)*phenol.* A mixture of 3-trifluoromethylaniline (17·7 g; 0·11 mol) and phenyl 5-methylsalicylate (22·8 g; 0·10 mol) was heated in an oil bath at 200° for 3 h. The product was allowed to cool to 150° and while still fluid was poured into EtOH from which it was recrystallized with charcoal treatment to give 19·2 g (65%) of pure product, m.p. 160–1°. Analysis: ($C_{15}H_{12}F_3NO_2$) C,H.

4-*Methyl*-2-(3-*trifluoromethylphenylcarbamoyl*)*phenoxyacetic acid.* To a solution of Na (1·19 g; 0·052 mol) in EtOH (130 ml) was added 4-methyl-2-(3-trifluoromethylphenylcarbamoyl)phenol (15·26 g; 0·052 mol) with stirring. To this solution was added ethyl chloroacetate (6·32 g; 0·052 mol) and the mixture was boiled under reflux for 7 h. The cool solution was diluted with H_2O (750 ml) and the crude ester was filtered, washed (H_2O) and dried.

The crude ester was dissolved in EtOH (180 ml) containing aqueous N NaOH (53 ml) and boiled under reflux for 7 h. The cold mixture was poured into water (500 ml) containing 5N HCl (15 ml) and the precipitate filtered, washed (H_2O) and dried. Recrystallization from n–Bu_2O afforded 10·7 g (58%) of pure cpd no. 26A as colourless needles, m.p. 225–6°. Analysis: ($C_{17}H_{14}F_3NO_4$) C,H,N.

Method 4

2-(3,4-*Dichlorophenylcarbamoyl*)-4-*ethylphenoxyacetic acid* (*cpd no.* 27*A*). 2-*Carboxy*-4-*ethylphenylacetate.* A solution of 2-carboxy-4-ethyl-phenol (50 g; 0·3 mol) in Ac_2O (150 ml) containing H_2SO_4 (0·1 ml) was heated at 70° for 4 h. The solution was concentrated *in vacuo*, poured into cold H_2O (1·5 litre) and the precipitated solid was ground, washed (H_2O) and dried to afford 59 g of crude 2-carboxy-4-ethylphenylacetate, m.p. 122–5°. Recrystallization from C_6H_6 gave 40 g (64%) of pure product as colourless needles, m.p. 135–7°.

2-(3,4-*Dichlorophenylcarbamoyl*)-4-*ethylphenylacetate.* A suspension of 2-carboxy-4-ethylphenylacetate (20 g; 0·096 mol) in $SOCl_2$ (14·5 ml; 0·2 mol) containing DMF (0·1 ml) was left to stand at room temperature for 16 h and finally was boiled under reflux for 1 h. The excess $SOCl_2$ was removed by co-distillation with several portions of C_6H_6 *in vacuo* and the residual red oil was dissolved in Me_2CO (100 ml). This solution was added during $\frac{1}{2}$ h with stirring to a solution of 3,4-dichloroaniline (16·2 g; 0·1 mol) and Et_3N (12·12 g; 0·12 mol) in Me_2CO (200 ml) and stirred for a further 4 h. The mixture was filtered, the filtrate was concentrated *in vacuo* to 50 ml and poured into 0·1N HCl (500 ml). The resulting oil slowly solidified and was filtered, washed (H_2O) and dried to give 29·5 g (87·5%) of crude product, m.p. 105–10°. Recrystallization from C_6H_6–light petroleum (40–60°) afforded 25·9 g (76·5%) of colourless needles, m.p. 112–4°. A portion recrystallized from C_6H_6 gave m.p. 115–7°. Analysis: ($C_{17}H_{15}Cl_2NO_3$) C,H,N.

2-(3,4-*Dichlorophenylcarbamoyl*)-4-*ethylphenol.* To a stirred suspension of 2-(3,4-dichlorophenylcarbamoyl)-4-ethylphenylacetate (25 g; 0·071 mol) in MeOH (75 ml)

was added a solution of KOH (5·35 g; 0·081 mol) in MeOH (75 ml) during ½ h and the mixture was stirred for 5 h. MeOH (100 ml) was removed *in vacuo* and the resulting oil was poured into 0·1N HCl (1 litre). The precipitate was filtered, washed (H_2O) and dried to afford 21·3 g (97%) of product, m.p. 180–4°. Recrystallization from Me_2CO–H_2O gave 17 g (77%) of the pure product as colourless rods, m.p. 185–7°. Analysis: ($C_{15}H_{13}Cl_2NO_2$) C,H,N.

2-(3,4-*Dichlorophenylcarbamoyl*)-4-*ethylphenoxyacetonitrile.* This compound was prepared by the reaction described under Method 2 to give colourless needles (90%), m.p. 138–40°. Analysis: ($C_{17}H_{14}Cl_2N_2O_2$) C,H,N.

2-(3,4-*Dichlorophenylcarbamoyl*)-4-*ethylphenoxyacetic acid.* To a solution of KOH (1·65 g; 0·025 mol) in MeOH (100 ml) was added 2-(3,4-dichlorophenylcarbamoyl)-4-ethylphenoxyacetonitrile (7·0 g; 0·02 mol) and the mixture was boiled under reflux for 5 h. The MeOH was removed *in vacuo* and the oil was poured into 0·1N HCl (250 ml). The precipitated solid was filtered, washed (H_2O) and dried to afford 7·0 g (95%) of product, m.p. 238–45°. Recrystallization from Me_2CO–H_2O gave 5·52 g (75%) of pure cpd no. 27A as colourless needles, m.p. 245–7°. Analysis: ($C_{17}H_{15}Cl_2NO_4$) C,H,N.

EXPERIMENTAL PHARMACOLOGY

Methods

Acute toxicity. Male albino mice, Smith & Nephew Research (SNR) strain, 25–30 g, 4 animals/dose were given the test compounds by mouth or intraperitoneally.

Table 3. *Intermediates not listed in the literature.*

Intermediate for compound	Compound	m.p.[1] °C	Formula	Analyses[2]
1T	2-Benzamidophenoxyacetonitrile	130–1	$C_{15}H_{12}N_2O_2$	C,H,N
2T	2-(4-Methylbenzamido)phenoxyacetonitrile	129–30	$C_{16}H_{14}N_2O_2$	C,H,N
3T	2-(3,4-Dichlorobenzamido)phenoxyacetonitrile	143–4	$C_{15}H_{10}Cl_2N_2O_2$	C,H,N
4T	2-(4-Chlorobenzamido)-4-chlorophenoxyacetonitrile	156–7	$C_{15}H_{10}Cl_2N_2O_2$	C,H,N
5T	4-Chloro-2-(3,4-dichlorobenzamido)phenoxyacetonitrile	163–4	$C_{15}H_9Cl_3N_2O_2$	C,H,N
6T	2-(4-Chlorobenzamido)-4-methylphenoxyacetonitrile	146–7	$C_{16}H_{13}ClN_2O_2$	C,H,N
7T	2-(3,4-Dichlorobenzamido)-4-methylphenoxyacetonitrile	160–1	$C_{16}H_{12}Cl_2N_2O_2$	C.H,N
8T	4-Methyl-2-(3-trifluoromethylbenzamido)phenoxyacetonitrile	145–6	$C_{17}H_{13}F_3N_2O_2$	C,H,N
9T	2-(4-Chlorobenzamido)-5-methoxyphenoxyacetonitrile	152–3	$C_{16}H_{13}ClN_2O_3$	C,H,N
10T	2-(3,4-Dichlorobenzamido)-5-methoxyphenoxyacetonitrile	159–60	$C_{16}H_{12}Cl_2N_2O_3$	C,H,N
11T	2-Phenylcarbamoylphenoxyacetonitrile	155–6	$C_{15}H_{12}N_2O_2$	C,H,N
12T	2-(3,4-Dichlorophenylcarbamoyl)phenoxyacetonitrile	148–9	$C_{15}H_{10}Cl_2N_2O_2$	C,H,N
13T	2-(3,5-Dichlorophenylcarbamoyl)phenoxyacetonitrile	179–80	$C_{15}H_{10}Cl_2N_2O_2$	C,H,N
14A	2-(3-Trifluoromethylphenylcarbamoyl)phenol	184–6	$C_{14}H_{10}F_3NO_2$	C,H
14T	2-(3-Trifluoromethylphenylcarbamoyl)phenoxyacetonitrile	131–2	$C_{16}H_{11}F_3N_2O_2$	C,H,N
15T	2-(4-Methoxyphenylcarbamoyl)phenoxyacetonitrile	100–1	$C_{16}H_{14}N_2O_3$	C,H,N
16T	4-Chloro-2-phenylcarbamoylphenoxyacetonitrile	116–7	$C_{15}H_{11}ClN_2O_2$	C,H,N
17T	4-Bromo-2-phenylcarbamoylphenoxyacetonitrile	132–3	$C_{15}H_{11}BrN_2O_2$	C,H,N
18T	4-Methyl-2-phenylcarbamoylphenoxyacetonitrile	182–3	$C_{10}H_{14}N_2O_2$	C,H,N
19A	4-t-Butyl-2-phenylcarbamoylphenylacetate	170–2	$C_{19}H_{21}NO_3$	C,H,N
	4-t-Butyl-2-phenylcarbamoylphenol	169–70	$C_{17}H_{19}NO_2$	C,H,N
19T	4-t-Butyl-2-phenylcarbamoylphenoxyacetonitrile	128–30	$C_{19}H_{20}N_2O_2$	C,H,N
20T	4-Chloro-2-(3,4-dichlorophenylcarbamoyl)phenoxyacetonitrile	153–4	$C_{15}H_9Cl_3N_2O_2$	C,H,N
21T	4-Chloro-2-(4-methylphenylcarbamoyl)phenoxyacetonitrile	142–3	$C_{16}H_{13}ClN_2O_2$	C,H,N
22A	4-Chloro-2-(3-trifluoromethylphenylcarbamoyl)phenol	195–6	$C_{14}H_9ClF_3NO_2$	C,H,N
22T	4-Chloro-2-(3-trifluoromethylphenylcarbamoyl)phenoxyacetonitrile	127–8	$C_{16}H_{10}ClF_3N_2O_2$	C,H,N
23A	4-Bromo-2-(3-trifluoromethylphenylcarbamoyl)phenol	205–6	$C_{14}H_9BrF_3NO_2$	C,H,N
23T	4-Bromo-2-(3-trifluoromethylphenylcarbamoyl)phenoxyacetonitrile	142–3	$C_{16}H_{10}BrF_3N_2O_2$	C,H,N
24T	2-(4-Chlorophenylcarbamoyl)-4-methylphenoxyacetonitrile	133–4	$C_{16}H_{13}ClN_2O_2$	C,H,N
25A	2-(3,4-Dichlorophenylcarbamoyl)-4-methylphenol	212–3	$C_{14}H_{11}Cl_2NO_2$	C,H
25T	2-(3,4-Dichlorophenylcarbamoyl)-4-methylphenoxyacetonitrile	141–2	$C_{16}H_{12}Cl_2N_2O_2$	C,H,N
26A	4-Methyl-2-(3-trifluoromethylphenylcarbamoyl)phenol	160–1	$C_{15}H_{12}F_3NO_2$	C,H
26T	4-Methyl-2-(3-trifluoromethylphenylcarbamoyl)phenoxyacetonitrile	129–30	$C_{17}H_{13}F_3N_2O_2$	C,H,N
27A	2-Carboxy-4-ethylphenylacetate	135–7	$C_{11}H_{12}O_4$	—
	2-(3,4-Dichlorophenylcarbamoyl)-4-ethylphenylacetate	115–7	$C_{17}H_{15}Cl_2NO_3$	C.H,N
	2-(3,4-Dichlorophenylcarbamoyl)-4-ethylphenol	185–7	$C_{15}H_{13}Cl_2NO_2$	C,H,N
27T	2-(3,4-Dichlorophenylcarbamoyl)-4-ethylphenoxyacetonitrile	138–40	$C_{17}H_{14}Cl_2N_2O_2$	C,H,N

[1] Melting points are uncorrected.
[2] All analytical results obtained for these elements were within ±0·4% of the theoretical values.

Approximate LD50 values were determined by inspection from mortalities occurring within 3 days.

PBQ writhing test. Female albino mice, SNR strain, four to six weeks old were injected with PBQ 35 min after the oral administration of the test compound. The mice were observed during the 5 min at which maximal writhing occurred in control animals. The number of writhes/mouse were counted and the dose which reduced the writhing rate by 50% was calculated from the dose response curves (10 mice/group) (Litchfield & Wilcoxon, 1949).

Rat foot oedema (carrageenan) test. A modification of the method of Winter, Risley & Nuss (1962) was used. The initial foot volume of the rats was determined volumetrically. A suspension of carrageenan (0·1 ml of 1% in normal saline) was injected subcutaneously into the plantar region of the right hind paw 1 h after the test compounds at 50 mg/kg or 10% gum acacia (controls) had been administered orally. Three h later the foot volume was measured again and the volume of the oedema determined. Results were calculated as percentage inhibition related to the control oedema volume. The oedema volume in control and treated animals was compared using students *t*-test.

Statistics. The PBQ 50% doses in μmol/kg were ranked in ascending order and each result also allotted to one of two groups A or B according to whether the corresponding rat foot result for the compound was positive or negative. The Kruskal-Wallis one-way analysis of variance by ranks (Siegel, 1956) was used to estimate the probability that groups A and B were from the same population.

Compounds no. 12T (SNR.2337) and 22T (SNR.2420) were selected for further evaluation by the cotton wool pellet granuloma test (Winter & Porter, 1957) fever induction (Brownlee, 1939), tail pinch analgesic (Bianchi & Franchescini, 1954) and ultraviolet erythema test (Winder, Wax & others, 1958).

RESULTS AND DISCUSSION

The degree of activity in the PBQ test varied from the low level of aspirin (110 mg/kg) and phenylbutazone (100 mg/kg) as shown by compounds 1A, 4A and 15A to the most potent derivatives (compounds 12T, 13T and 22T) which were approximately 50 times more active. The most potent of these (compound 22T) had the same order of activity in the writhing test as indomethacin. Although active anti-inflammatory agents consistently produce significant reductions in rat foot volume, the absolute values for the percentage reductions vary from day to day. For this reason the results in this test have been expressed as + (active) or − (inactive) corresponding to significant levels of $P = <0{\cdot}05$ and $>0{\cdot}1$ respectively.

Kruskal–Wallis one-way analysis of variance by ranks gave a value of $P = 0{\cdot}001$ indicating that the rat foot test +ve group ranked by the PBQ results did not come from the same population as the rat foot test −ve group similarly ranked. This evidence in addition to that discussed in a previous paper supports the assumption that in this series the PBQ results indicate anti-inflammatory activity, and results in the latter tests were used to guide the synthetic program.

Correlations between structure and activity for the series of acids in Table 1 were discussed previously. Correlations in the other series and the differences in activity between acid-tetrazole pairs are discussed with reference to ring P and ring Q.

Series in Table 1

Little correlation between structure and activity was apparent within the ten pairs

of compounds in this series. Insufficient compounds are presented to illustrate a preferred substituent for either ring P or ring Q.

Series in Table 2

The introduction of substituents into ring P with no substituent in ring Q caused increases in activity in the acid series but not in the tetrazole series. The introduction of substituents into both rings led to more complicated effects not explained simply in terms of the different π constants.

The most active tetrazoles were compounds 12T, 13T, 20T and 22T with PBQ writhing figures of 5·8, 7·4, 8·5 and 3·5 μmol/kg respectively and the most active acids in this test, compounds 17A and 22A, gave figures of 8·6 and 5·4 μmol/kg. All of these compounds were from Table 2. Several of these more active compounds were examined by other anti-inflammatory procedures and compounds 12T (SNR.2337) and 22T (SNR.2420) were selected for further study.

Table 4 gives results of these further tests.

Table 4. *Results obtained with compounds* 12*T* (*SNR*.2337) *and* 22*T* (*SNR*.2420) *in five anti-inflammatory tests and one analgesic test, in comparison with indomethacin and phenylbutazone.*

Test	Compound 12T (SNR.2337)	Compound 22T (SNR.2420)	Indomethacin	Phenylbutazone
PBQ (50% dose mg/kg oral) ..	2·1	1·4	1·3	100
Rat foot oedema (threshold dose mg/kg oral)	10	8	<1·0	10
Antipyresis in rats[1] (temperature index[2] at 30 mg/kg, oral) ..	1·0	1·0	—	0·62
Mouse tail pinch analgesic test[3]	−ve at 320 mg/kg oral	−ve at 200 mg/kg oral	—	—
Cotton wool pellet[4] granuloma test (threshold dose mg/kg oral)	12	12	0·33	10
Guinea-pig, erythema test (threshold dose mg/kg oral) ..	40–80	40–80	—	20

[1] Brownlee (1939).
[2] Winter, Risley & Nuss (1963).
[3] Bianchi & Franchescini (1954).
[4] Winter & Porter (1957).
[5] Winder & others (1958).

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Potential irreversible inhibitors of acetylcholinesterase: *N*-trimethyl-*N'*-iodoacetyl diaminoalkane iodides

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Potential irreversible inhibitors of acetylcholinesterase based on *N*-trimethyl-*N'*-iodacetyl diaminoalkane iodide $Me_3\overset{+}{N}[CH_2]_n$-$NHCO{\cdot}CH_2I{\cdot}I^-$, (I; n = 4) and (II; n = 2) have been prepared. (I) and (II) are both competitive inhibitors of the enzyme with K_i, $5{\cdot}9 \times 10^{-4}$M and $1{\cdot}2 \times 10^{-3}$M respectively. (I) is a partial agonist and (II) is a true agonist at the muscarinic receptor of guinea-pig ileum.

Many examples are known where a substrate or reversible competitive inhibitor of an enzyme when modified by the attachment of a suitable alkylating group becomes a specific irreversible inhibitor of the enzyme (Baker, 1964; Singer, 1967). The modified molecule is specifically bound at the active site by intermolecular forces and is correctly positioned for covalent bond formation with a functional group at (or near) the active site. We have designed compounds which are structurally related to acetylcholine based on *N*-trimethyl-*N'*-iodoacetyl diaminoalkane iodide, $Me_3\overset{+}{N}$-$[CH_2]_n{\cdot}NHCO{\cdot}CH_2I{\cdot}I^-$, (I; n = 4) and (II; n = 2) as potential irreversible inhibitors of acetylcholinesterase. The pharmacological action of (I) and (II) at the muscarinic receptor of guinea-pig ileum was also examined.

METHODS AND RESULTS

N-*Trimethyl*-N'-*iodoacetyl-1,4-diaminobutane iodide* (*I*; *n* = 4). Ethyl acetate (30·65 ml) was refluxed with 1,4-diaminobutane (85 g) for 48 h. The resulting mixture was distilled to give an oil (36·6 g), b.p. 119°/0·5 mm. Exposure of the oil in a thin film to air gave a white powder, m.p. 125·5–128°, which on sublimation gave white crystals, m.p. 132–133° of N-*acetyl-1,4-diaminobutane hemihydrate*. (Found; C, 52·0: H, 10·6; N, 19·9. $C_6H_{14}ON_2.\frac{1}{2}H_2O$ requires C, 51·8; H, 10·9; N, 20·1%). ν_{max} (KBr), 3300 (NH), 1670 (C=O) cm^{-1}.

N-Acetyl-1,4-diaminobutane (5 g) in ethanol and iodomethane (9·5 ml) were added simultaneously to a stirred solution of sodium hydroxide (3·56 g) in ethanol at 0°. After the vigorous reaction had ceased, the mixture was maintained at 45–50° with stirring for 0·5 h and then kept overnight at 4°. The mother liquors were cropped and the organic material recrystallized from acetone to give white crystals (3·7 g), m.p. 179–179·5° of N-*trimethyl*-N'-*acetyl-1,4-diaminobutane iodide*. (Found: C, 36·3; H, 6·9; N, 9·5; I, 41·9. $C_9H_{21}ON_2I$ requires C, 36·0; H, 7·05; N, 9·3; I, 42·3%) ν_{max} (KBr), 3300 (NH), 1670 (C=O) cm^{-1}.

N-Trimethyl-*N'*-acetyl-1,4-diaminobutane iodide (1 g) was refluxed with alcoholic sodium hydroxide (25 ml, 1·4 g) for 5 h. The resultant oil was removed and refluxed with an excess of hydriodic acid for 0·25 h to give a reddish-brown solid which on

recrystallization from ethanol gave yellow crystals (0·52 g), m.p. 248–249° of N-*trimethyl-1,4-diaminobutane hydriodide iodide,* (Found; C, 21·60; H, 5·33; N, 7·40; I, 66·00. $C_7H_{20}N_2I_2$ requires C, 21·77; H, 5·22; N, 7·26; I, 65·73%). ν_{max} (KBr), 3450 (NH) cm^{-1}.

An alcoholic solution of sodium hydroxide (0·22 g) was added to a stirred solution (50 ml) of *N*-trimethyl-1,4-diaminobutane hydriodide iodide (1 g) in ethanol. Iodoacetyl chloride (1·06 g) was added to the cooled solution and the mixture stirred at 0° for 0·5 h. The mother liquors were cropped and the solid material obtained recrystallized from ethanol to give creamy-white crystals (0·48 g), m.p. 147–147·5° of N-*trimethyl*-N'-*iodoacetyl-1,4-diaminobutane iodide* (I). (Found: C, 25·4; H, 4·7; N, 6·3; I, 58·7. $C_9H_{20}ON_2I_2$ requires C, 25·4; H, 4·7; N, 6·6; I, 59·6%). ν_{max} (KBr), 3250 (NH), 1670 (C=O) cm^{-1}.

N-*Trimethyl*-N'-*iodoacetyl-1,2-diaminoethane iodide.* (*II*; $n = 2$). (II) was prepared from ethylenediamine by the general method described above and had m.p. 176–177°, (Found: C, 21·3; H, 4·1; N, 7·2; I, 63·9. $C_7H_{16}ON_2I_2$ requires C, 21·1; H, 4·05; N, 7·0; I, 63·8%).

(I) and (II) as irreversible inhibitors of acetylcholinesterase. The general inhibition procedure and the materials used have been described elsewhere (Beddoe & Smith, 1971).

A solution (19 ml) of each compound (1×10^{-2}M) was incubated with acetylcholinesterase (1 ml, 3·6 mg) in a medium containing sodium chloride (0·2M) at both pH 6·5 and pH 9·5. Samples (2 ml) of the mixture were periodically withdrawn and the remaining enzyme activity determined.

There was no decrease in the enzyme activity after incubation with (I) or (II) for 21 h at either pH.

(I) and (II) as reversible inhibitors of acetylcholinesterase. The rates of hydrolysis of acetylcholine over the concentration range $4\text{–}12 \times 10^{-4}$M by acetylcholinesterase (0·36 mg) were determined in a constant final reaction volume (20 ml) containing sodium chloride (0·2M) at pH 7·0 and 25°. The experiment was repeated in the presence of either (I) or (II) (2×10^{-3}M). The results were plotted according to Lineweaver & Burk (1934), i.e. 1/v *vs* 1/[S] where v is the rate of hydrolysis of the substrate (S).

The two linear curves obtained for each experiment in the presence and absence of the inhibitor intersected at the same point on the 1/v axis showing that each compound was a competitive inhibitor of the enzyme. The K_i values obtained for (I) and (II) were $5·9 \times 10^{-4}$ and $1·2 \times 10^{-3}$M respectively and the K_m value obtained under identical conditions but in the absence of the inhibitor was $1·65 \times 10^{-4}$M (*cf.* 2×10^{-4}M, Wright & Sabine, 1948).

Pharmacological action of (I) and (II) on guinea-pig isolated ileum. The tissue was employed as described previously using the Sanborn transformer and recorder (Beddoe, Nicholls & Smith: unpublished observations).

A cumulative dose-response curve for acetylcholine was initially obtained and then the response of the tissue to (I) and (II) was separately observed. The maximal heights of contracture of the tissue obtained with (I) and (II) were 60 and 100% respectively of the maximal contracture for the tissue with acetylcholine, suggesting that (I) is a partial agonist and (II) a true agonist at the muscarinic receptor of guinea-pig ileum. The mean value of the equipotent molar ratio for (II) by comparison with acetylcholine was 284.

DISCUSSION

(I) and (II) are reversible competitive inhibitors of acetylcholinesterase. The inhibition constants, K_i, were $5{\cdot}9 \times 10^{-4}$M and $1{\cdot}2 \times 10^{-3}$M respectively so that (I) is more firmly bound at the active site of the enzyme than (II).

This is to be expected since the higher homologue (I) possesses additional hydrophobic binding forces (Belleau, 1967). The absence of marked inhibition by compound (II), which structurally closely resembles acetylcholine, could be due to interaction with the anionic site and with a region away from the esteratic site. This view is supported by the similarity in the K_i values of (II) and tetramethylammonium ($1{\cdot}87 \times 10^{-3}$M, Kellett & Doggett, 1966; $2{\cdot}33 \times 10^{-3}$M, Belleau, 1965) which binds only to the anionic site.

Although both (I) and (II) are bound at the active site in an enzyme-inhibitor (EI) complex, neither alkylates a nucleophilic group on the enzyme in its vicinity with irreversible inhibition of the enzyme. The iodoacetyl group is a strong electrophile and is known to alkylate a variety of functional groups on proteins (Baker, 1964) so it would seem that in the EI-complex there is not a suitable or correctly positioned nucleophile on the enzyme in the vicinity of the iodoacetyl function for reaction.

O'Brien (1969) has recently indicated the presence of three binding sites for substrates and inhibitors of acetylcholinesterase designated α (anionic site), β and γ (hydrophobic sites), in addition to the esteratic site. In contrast to the results reported here, where binding by specific forces at the α-site is involved, we consider that the success of a wide range of chemical structures incorporating a phosphate or carbamate function as irreversible inhibitors at the esteratic site of the enzyme is due to binding at β- or γ-sites by non-specific van der Waal's and hydrophobic forces. The non-specificity of these binding forces would allow some degree of manoeuvrability in the location by these compounds of the required nucleophile (serine) on the enzyme surface (*cf.* Smith & Williams, 1967).

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Sulphamethoxydiazine crystal forms

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Methods of preparation of three polymorphs, two solvates and an amorphous modification of sulphamethoxydiazine are described. Infrared spectroscopy and X-ray diffraction data are given for the characterization of the various forms. The interconversion of these forms under various conditions of heating, suspension in water and grinding is studied. The dissolution behaviour of the different forms is also discussed. All forms on heating to 150° change to the same form and on suspension in water change to another water-stable form.

Physical stability and biological availability have been shown to be greatly affected by polymorphism and solvation of drugs (Higuchi, 1958; Frederick, 1961; Higuchi, Lau & others, 1963; Shefter & Higuchi, 1963; Aguiar, Krg & others, 1967). The pharmaceutical applications of polymorphism have been recently reviewed by Haleblian & McCrone (1969). These papers suggested that physical chemical studies of solid drugs are essential before their formulation in different dosage forms.

Sulphamethoxydiazine was reported by Svatek, Knobloch & Budesinsky (1966) to be dimorphic. Methods of preparation of the two crystal forms were described. Later, Mesley & Houghton (1967) described methods for the preparation of one amorphous and three crystalline forms of the same compound. Because of the different number of polymorphs, and the difficulty of correlating their preparation and characterization, more thorough investigation of this polymorphic system seemed necessary.

We have examined the preparation, characterization, interconversion and dissolution behaviour of the various crystal forms of sulphamethoxydiazine.

METHODS AND RESULTS

Materials and apparatus

Sulphamethoxydiazine was supplied by courtesy of Alexandria Co. for Pharmaceuticals and Chemical Industries, U.A.R. The purity of the starting material and products of crystallization from different solvents was checked by paper chromatography using the solvent system described by Steel (1951). Solvents used for crystallization were of B.P. quality.

Infrared spectra were measured with a Perkin-Elmer double-beam grating infrared spectrophotometer model 237B and the concentration of sulphamethoxydiazine during dissolution rate studies was followed by ultraviolet spectrophotometry. X-ray diffraction measurements were made with a General Electric XRD-6 diffractometer with variables: 3° beam slit and 0·2° detector slit; CuK_α radiation, 45 KV, 11 mA, Ni filtered.

Preparation of the different crystal forms

The general procedure for the preparation of the different crystal forms involved

crystallization from specific solvents. For this purpose, about 0·2 g of the drug was dissolved in a suitable volume of an appropriate solvent to form a saturated solution at the boiling point of that solvent. The solution was allowed to cool slowly and stand at room temperature, except for Form II, for 24 h. The crystals which separated were then filtered on a sintered-glass disc (Jena 39 G 3), dried in a current of air at room temperature (25°) and stored in a desiccator. Optimum conditions for the preparation of the different crystal forms are summarized as follows:

Form I was prepared by crystallization from boiling water or by heating any other form to 150°.

Form II was prepared by rapid cooling in a refrigerator of a saturated solution in ethanol.

Form III was prepared by crystallization from any of the following solvents: methanol, isopropanol, ethyl acetate or by precipitation from a solution in acetone by the addition of water.

Forms IV and V were prepared by crystallization from dioxane and chloroform respectively.

An amorphous form was also prepared by melting any of the previous crystal forms and slow cooling of the melt.

Characterization of the different crystal forms

The main differences observed in infrared spectra of Forms I–V and the amorphous form in Nujol mulls are summarized in Table 1. The X-ray diffraction patterns of the various crystal forms are shown in Fig. 1, A–E. Such patterns show distinct differences that can be used for their characterization.

Interconversion of the different crystal forms

(a) *Crystallization.* Any form can be converted to another by crystallization from the appropriate solvent as described under "Preparation of the different crystal forms".

Table 1. *Characterization of sulphamethoxydiazine crystal forms by infrared spectroscopy*[(a)].

Form	800–875 cm^{-1}	900–970 cm^{-1}	1550–1600 cm^{-1}	3000–3500 cm^{-1}	Other characteristic bands and absorbance ratios (r)
I	b (838) & 2s§	b (927) & 2s§	2b (1567, 1595)	2b (3345, 3458)	Consistent s (1640) A 943/A 785 = 1·72 A 1320/A 1345 = 1·82
II	doublet (835, 852)	2b (922, 950)	doublet (1580, 1595)	2b (3275, 3365)	Relatively strong b (922) A 922/A 950 = 0·53 A 1580/A 1595 = 0·98
III	b (845) & 2s§	2b (903, 935)	2b (1567, 1595)	broad b (3190), b (3315), b (3398) & 2s§	b (980) A 980/A 785 = 0·43 A 1310/A 1323 = 0·69
IV	2b (830, 873)	b (944) & s	b (1595) & s	2b (3343, 3453)	b (1120) Relatively strong b (1253) A 830/A 873 = 0·66 A 1253/A 785 = 1·60
V	b (832) & s	b (940) & 2s*	b (1595) & 2s*	2b (3350, 3455)	Strong b (755) A 755/A 785 = 3·29 A 832/A 862 = 1·75
Amorphous	b (830) & s	broad b (935)	2b (1567, 1595)	2b (3335, 3438)	—

(a) Figures between brackets denote the frequency in wavenumbers at peak maxima; (b) = band; (s) = shoulder, § on either side of the band, * on one side of the band; (r) = ratio of absorbance (A) of the specified bands.

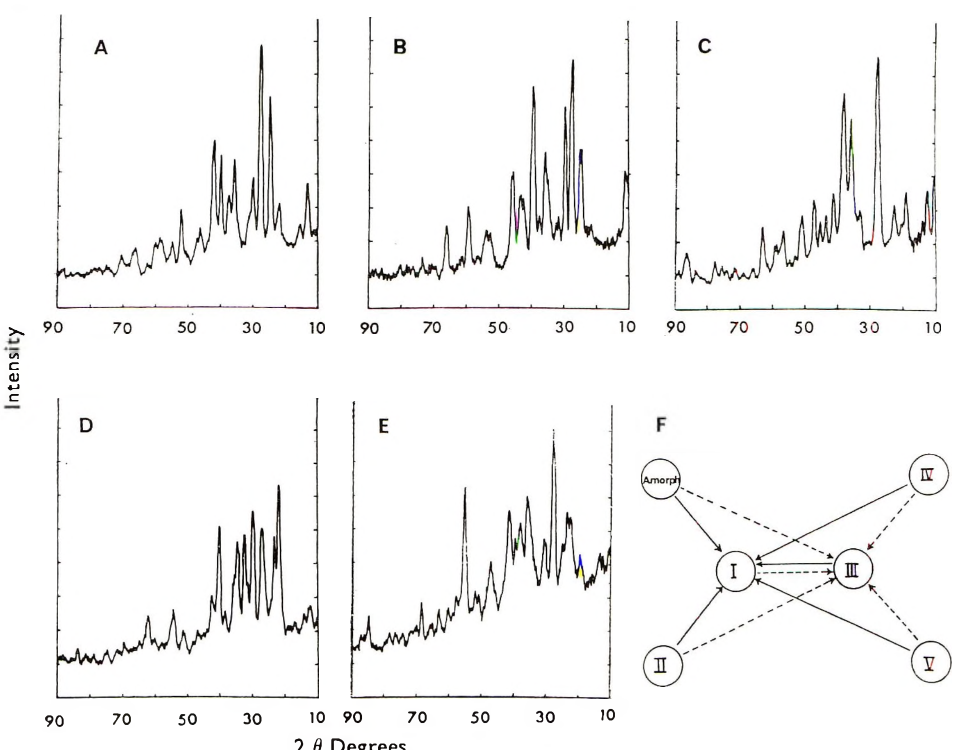

FIG. 1. A–E. X-ray diffraction data of sulphamethoxydiazine crystal forms.
F. Interconversion of sulphamethoxydiazine crystal forms. —— heating to 150°, - - - - grinding or suspension in water.

(b) *Heating.* Heating to 150° was found to effect transformation from any form to Form I. A similar procedure was suggested by Mesley & Houghton (1966) for identification purposes.

(c) *Suspension in water.* Suspension of all forms in water resulted in transformation to Form III. The time necessary to achieve such a transformation varied according to the sample source and particle size. Forms IV, V and the amorphous form were found to change initially to Form I; prolonged suspension, however, completed the transformation to Form III. Form II, on the other hand, changed in a relatively short time to Form III.

(d) *Grinding.* Dry grinding converted all forms to Form III. Form I was detected as an intermediate in all cases. Grinding under water was found to accelerate the transformation to Form III.

A summary of the course of interconversions is illustrated in Fig. 1F.

Dissolution rate studies

The procedure adopted for measuring the dissolution rates of the different forms consisted in suspending excess quantities of the solid (screened to a particle size of 80–90 μm) in 200 ml of 0·1N HCl in a 250 ml glass stoppered flask. The flask was then rotated at 70 rev/min in a constant temperature water-bath maintained at 30° ±0·1°. At measured time intervals, 3 ml aliquots were withdrawn and immediately filtered through a Jena 39 G 3 sintered-glass funnel, 3 ml of 0·1N HCl were replaced

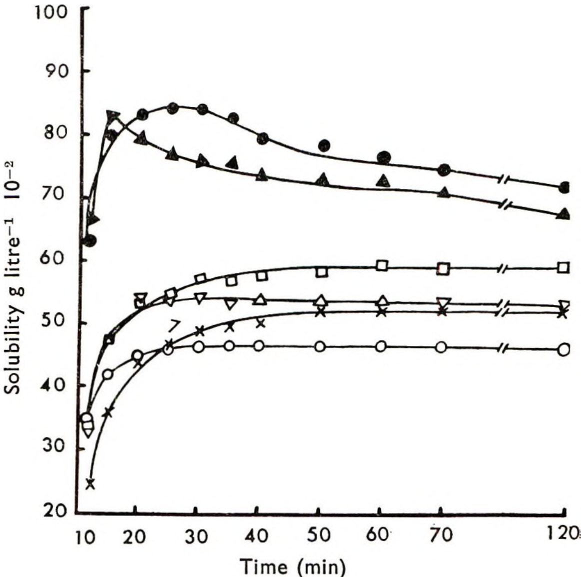

FIG. 2. Dissolution rates of sulphamethoxydiazine crystal forms. ×—× Form I, ●—● Form II, ○—○ Form III, □—□ Form IV, △—△ Form V, ▲—▲ amorphous form.

in the flask. Appropriate dilutions were made and the concentration of the drug in the various samples was determined by measuring its ultraviolet absorbance at 228 nm and referring to a standard curve. Results of the dissolution rate measurements of the various forms are shown in Fig. 2.

DISCUSSION

Sulphamethoxydiazine exists in the solid state in one of five or more crystal forms or in an amorphous state. Forms I, II and III represent a true polymorphic system, whereas Forms IV and V are probably solvates of dioxane and chloroform respectively.

We found the methods of preparation of sulphamethoxydiazine crystal forms (Svatek & others, 1966; Mesley & Houghton, 1967) in many cases difficult to reproduce. Crystallization from aqueous ethanol, reported by Mesley & Houghton (1967) to produce Form A gave Form III. Crystallization from aqueous solution reported by the same authors to produce Form B in our hands resulted in Form I. Had Mesley & Houghton (1967) suggested that their Form A changed to Form B by heating this would have matched the change we found from Form III to Form I; unfortu'nately the contrary was reported. The procedure of precipitating sulphamethoxydiazine from acetone solution by the addition of water or from a solution in an alkali by the addition of an acid gave Form III. This too was contrary to the findings of Mesley & Houghton (1967) who reported the production of a third Form C. We found their procedure in most cases to result in mixtures of crystal forms and amorphous material.

Form α (m.p. 212°) prepared by Svatek & others (1966) by "rapid motion crystallization from warm aqueous solutions" was found to match Form I on basis of similarity of their infrared spectra. Their β form (m.p. 197°) was prepared from "saturated aqueous solutions at room temperature". They also recommended crystallization

from acetone for the preparation of the pure β form. This latter procedure was found to give Form III. The infrared spectrum published for the β form is identical with that of our Form III.

All forms had a melting point of 212–214°. No other transitions were observed below the melting point except when solvent was given off; e.g. Forms IV and V in which case the crystals turned opaque. This is not in agreement with the finding of Svatek & others (1966) who reported a m.p. of 197° for their β-form. The change to the α-form by heating to 197° (described as melting of the β-form and slow cooling of the melt) was matched in the present study by a change to Form I. However, the description of this temperature (197°) as a melting point of the β-form does not agree with our finding that all forms change to Form I at 150° and consequently should have the same melting point. Evidence is gradually accumulating against the general belief reported by Haleblian & McCrone (1969), that polymorphs have different melting points. Among several drug systems recently studied, e.g. cortisone acetate (Carless, Moustafa & Rapson, 1966), some sulphonamides (Mesley & Houghton, 1967; Moustafa & Carless, 1969) and the antiviral compound SK & F 30097 (Ravin, Shami & Rattie, 1970), all polymorphs were found to change to one and the same form before melting and therefore to have the same melting point.

Infrared spectra of the various forms in Nujol mulls could be used conveniently in their characterization. Comparison of these spectra suggest that the most probable type of association characterizing the various crystal forms is that involving intermolecular hydrogen bonding. This is likely to take place between the aromatic amino-group of one molecule and the oxygen of the sulphonamide linkage of another. Evidence supporting this suggestion is manifested by a lowering of frequency of the NH stretching vibrations which is particularly obvious in the infrared spectrum of Form II (Bellamy, 1964). The absorption bands corresponding to these vibrations appear at the lower frequencies of 3275, 3365 cm^{-1} in the infrared spectrum of Form II as compared to 3345, 3458 cm^{-1} in case of Form I.

A relatively strong band at 1253 cm^{-1} with another band at 1120 cm^{-1} and a strong band at 755 cm^{-1} in the infrared spectra of Forms IV and V suggest their presence as solvates of dioxane and chloroform respectively (Cross, 1960). A similar observation was described by Mesley (1965) and Cords (1953) for chloroform adducts of certain steroids.

Many of the differences in the infrared spectra of the crystal forms appear in the Fingerprint region. Correlation of such differences to the mode of association between molecules in the crystal lattice of the various forms is rather difficult. However, the evidence available so far in this study confirms the opinions of Mesley & Houghton (1967) and Svatek & others (1966) who excluded amide-imide tautomerism as an explanation of the differences observed in the infrared spectra of sulphamethoxydiazine crystal forms.

Dissolution rate studies of the various crystal forms (Fig. 2) showed obvious differences in solubility and dissolution rate between Forms I, III, IV and V on one hand and Form II and the amorphous form on the other. The latter forms showed an apparent equilibrium solubility (viz. peak of the dissolution rate curve) which is about 1·8 times that of the water-stable Form III. However, longer periods of contact of the solid with the dissolution medium was found to result, as expected, in a decrease in solubility to a value corresponding to that of the water-stable Form III. Infrared determinations confirmed the transformation of the various forms to

Table 2. *Thermodynamic values calculated for Forms II and III of sulphamethoxydiazine*

Crystal form	Trans. temp. °C	Heat of solution ΔH cal/mol	ΔG_{303} cal/mol	ΔS_{303} e.u.	ΔS e.u.
III	—	−6530	—	—	—
II	104	−5111	−291	−3·7	−3·8

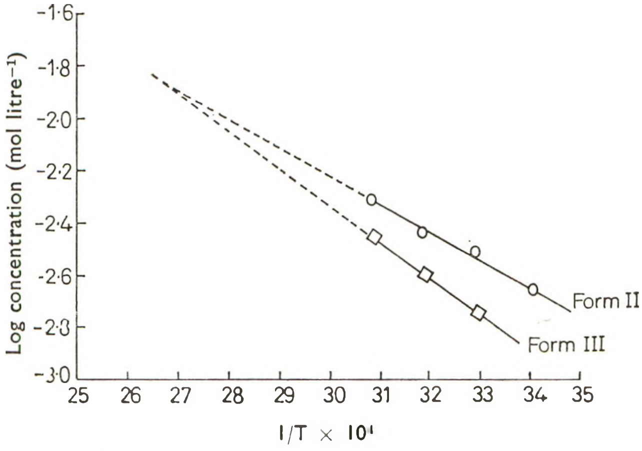

FIG. 3. The van't Hoff-type plot for Forms II and III of sulphamethoxydiazine. ○—○ Form II, □—□ Form III.

Form III. The apparent solubilities of Forms II and III determined at various temperatures were plotted according to the classical van't Hoff plot (Shefter & Higuchi, 1963; Higuchi & others, 1963; Aguiar & others, 1967; Poole & Bahal, 1970). Results of this treatment are shown in Fig. 3 and Table 2. The observed entropy difference might be related to the possible association by intermolecular hydrogen bonding as already outlined. The thermodynamics of the solid state transformation of Form II to Form III is particularly important since Form II has the highest solubility of all crystal forms and would therefore be the one favoured for pharmaceutical use. Its transformation to the least soluble water-stable Form III is an important factor in deciding its physical stability and biological availability from various dosage forms.

From the previous discussion, it could be suggested that Form I is a suitable reference material for the identification of sulphamethoxydiazine. Heating any form to 150° would be the only prerequisite.

In four commercial preparations (tablets and suspensions) of sulphamethoxydiazine, Form I or mixtures of Forms I and III were encountered. Results of the present investigation would recommend the use of Form II in pharmaceutical preparations provided that adequate measures are taken to keep it in this metastable state.

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LETTERS TO THE EDITOR

Correlation of analgesic potencies of *N*-substituted normeperidines and *in vitro* *N*-dealkylation

Structural variation of the substituent attached to the basic nitrogen in strong analgesics may cause substantial potency variations (Portoghese, 1965, 1966). It is not known whether the potency differences are a reflection of different drug concentrations in the biophase or to differing receptor affinities or a combination of these. Since it has been reported (Hardy, Lister & Stern, 1965; Christian, Gorodetzky & Lewis, 1971) that increasing the chain length of the *N*-substituent in normeperidine derivatives gives rise to large differences in ED50's, we have studied the *in vitro* *N*-dealkylation of homologues in this series (methyl to nonyl) in an effort to ascertain whether metabolism can be correlated with the structure-activity relations.

Enzymatic dealkylation of *N*-substituted normeperidines was examined in mouse liver homogenate. Equimolar concentrations of the compounds were individually incubated in air with the 9000 *g* supernatant of mouse liver homogenate for 60 min at 37°. The incubation mixture contained NADP and NADPH generating system in a pH 7·4 phosphate buffer.

The extent of *N*-dealkylation was determined by measuring the normeperidine produced using a g.l.c. assay. The normeperidine extracted from the basified incubation medium was mixed with 4-phenyl-4-piperidinol (internal standard) and acetic anhydride. A Perkin-Elmer 900 gas chromatograph equipped with 6 ft × 0·25 in o.d. glass column packed with 3% OV-17 on chromosorb W and a flame ionization detector was used at a column temperature of 205° and N_2 as carrier gas. The amount of normeperidine was determined from the peak-height ratio of *N*-acetylnormeperidine (Rt 9 min) to that of *N*-acetyl-4-phenyl-4-piperidinol (Rt 15 min).

Table 1 shows the percentage substrate dealkylated as determined by the amount of normeperidine produced after 60 min incubation. All the compounds were substrates for the microsomal enzymes. It is of interest that there is no linear dependence of dealkylation on liposolubility as has been reported for demethylation of tertiary amines (McMahon, 1961; McMahon & Easton, 1961; Hansch, Steward & Iwasa, 1965).

The ED50 values were determined by the Eddy hot-plate method (Eddy & Leimbach, 1953) after intravenous administration in mice (Christian, Gorodetzky, &

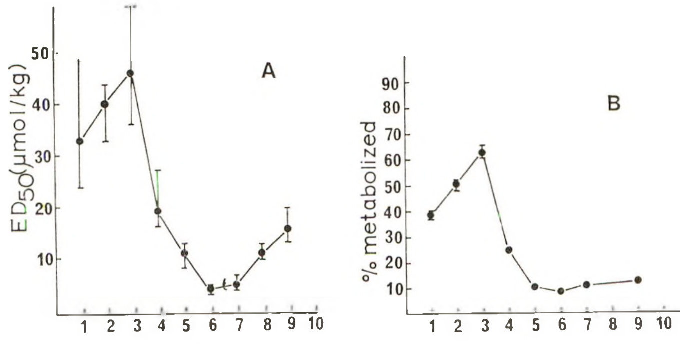

FIG. 1. Effect of chain length of the *N*-substituent on the analgesic activity and *in vitro* enzymatic *N*-dealkylation of meperidine congeners. One curve (A) represents ED50 as determined by the hot plate method in μmol/kg versus the number of carbons in the alkyl chain. The other curve (B) represents percent substrate *N*-dealkylated *in vitro* versus the number of carbons in the alkyl chain. Vertical lines represent 95% confidence limits in the hot plate graph and s.e. in the other graph.

Table 1. *Chain length of* N-*substituent and enzymatic* N-*dealkylation of meperidine congeners.*

Substrate[a] R=	Normeperidine produced[b] μmol (s.e.)	% Substrate *N*-dealkylated (s.e.)[b]
Methyl ..	0·192 (0·007)	38·4 (1·45)
Ethyl	0·255 (0·008)	51·0 (1·52)
n-Propyl ..	0·314 (0·012)	62·8 (2·43)
n-Butyl ..	0·124 (0·002)	24·8 (0·38)
n-Pentyl ..	0·053 (0·002)	10·6 (0·45)
n-Hexyl ..	0·043 (0·002)	8·6 (0·43)
n-Heptyl ..	0·055 (0·002)	11·0 (0·44)
n-Nonyl ..	0·061 (0·002)	12·4 (0·46)

a. Substrates used as hydrochloride salts in $1{\cdot}0 \times 10^{-4}$M concentration.

b. Each value is the mean of four experiments.

Lewis, 1971). Fig. 1 illustrates the remarkable similarities in the curves relating *N*-dealkylation and ED50 to chain length. Linear regression analysis of the data shows a high correlation ($r = 0{\cdot}978$) between *N*-dealkylation and ED50.

These results suggest that *N*-dealkylation is a major metabolic pathway for these compounds *in vivo*, and that the differences in analgesic potency between these congeners is due primarily to differences in their metabolism (and hence to differences of drug concentration in the biophase) rather than differences in their interaction with analgesic receptors.

We wish to thank Mrs. Jorunn Winsnes for technical assistance and Dr. Gorodetzky for giving us permission to use the ED50 data before publication. We gratefully acknowledge financial support for this research provided by Geigy Pharmaceuticals and Lilly Research Laboratories.

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July 26, 1971

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Failure of peristaltic stimulants to restore peristalsis previously blocked by 2,4-dinitrophenol

The action of metabolic inhibitors on the active transport of cations in nerve fibre (Hodgkin & Keynes, 1955), on mammalian striated muscle (Barnes & Duff, 1954) and on the electrical and mechanical activity of the smooth muscle of the guinea-pig taenia coli (Bülbring & Lüllman, 1957) have been described. We have attempted to relate the peristaltic block produced by 2,4-dinitrophenol (DNP) with active cationic transport in nerves or smooth muscles (or in both structures) involved in the peristaltic reflex.

A modified method of Trendelenburg (1917) to record the peristaltic activity (Beleslin & Varagić, 1958) was used. Volume changes in the intestinal segment were recorded by means of a float recorder and movements of the longitudinal muscle by an isotonic lever. The intestine was suspended in a 20 ml bath containing Tyrode solution at 36° gassed with oxygen. The peristaltic reflex was elicited by increasing the intraluminal pressure by 3–4 cm H_2O for about 90 s; the increase in pressure was kept constant throughout.

The effect on the peristaltic reflex of DNP was studied by introducing it (10–500 μg/ml) into the lumen of the isolated intestine or by adding it to the bath. By both routes DNP depressed or blocked the peristaltic reflex and this was reversible within 10–20 min. DNP in concentrations higher than 100 μg/ml contracted the longitudinal muscle and blocked the peristaltic reflex.

Peristaltic stimulants such as acetylcholine (500–600 μg/ml), arecoline (0·05–0·25 μg/ml), nicotine (100–200 μg/ml) and eserine (0·02–5·0 μg/ml) acting from the serosal side did not antagonize the inhibitory effect of DNP on the peristaltic reflex obtained either from mucosal or serosal side. When the peristaltic reflex was blocked by DNP, acetylcholine and eserine contracted the circular and longitudinal smooth muscles and caused a few small waves which were not typical peristaltic waves. In similar experiments serosal and mucosal application of 5-hydroxytryptamine (1–15 μg/ml) and histamine (1–50 μg/ml) failed to restore the peristaltic waves previously inhibited by DNP on either surface of the gut.

When the peristaltic reflex was depressed or abolished by serosal application of DNP, adenosinetriphosphate (ATP) and adenosinediphosphate (ADP) in concentrations from 10 to 250 μg/ml within 20 min failed to restore this reflex.

Serosal application of potassium chloride in concentrations from 1–4 mg/ml contracted both the longitudinal and circular smooth muscles, but failed to restore the peristaltic reflex previously blocked by serosal application of DNP. There was no restoration of the reflex, but a few small waves appeared which were not typical peristaltic waves. Calcium chloride and magnesium chloride were without effect on the peristaltic reflex previously blocked by serosal application of DNP.

It is evident that the peristaltic stimulants, ATP, ADP and cations cannot restore the peristaltic reflex previously blocked by DNP. Moreover, acetylcholine, eserine and potassium chloride contracted both longitudinal and circular smooth muscles in the presence of DNP. Evans, Schild & Thesleff (1958) have shown that drugs may activate the contractile elements of plain muscle without the mediation of membrane depolarization. The present experiments suggest that a contraction in smooth muscles may be produced by acetylcholine, eserine and potassium chloride in the presence of DNP when the active cationic transport is blocked. However, acetylcholine, eserine and potassium chloride cannot produce peristaltic waves when the active cationic transport is blocked, though both longitudinal and circular smooth muscles are contracted.

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Relaxing potency of terbutaline and orciprenaline on rat uterus

The inhibitory effects of the selective β-receptor stimulating agent terbutaline (Persson & Olsson, 1970) and of orciprenaline have been compared on carbachol-induced contractions in the rat isolated uterus.

Female rats, Sprague-Dawley, 130–150 g, had 0·2 μg 17-β-oestradiole benzoate subcutaneously 24 h before being killed by a blow on the head and bled. The middle part of each uterus horn, of about 2 cm length, was put in an organ bath (25 ml) containing calcium-poor Locke solution (45 g NaCl, 2·1 g KCl, 0·3 g $CaCl_2$, 2·5 g $NaHCO_3$, 2·5 g glucose in 5 litre of glass-redistilled water) at 25° and gassed with 5% carbon dioxide in oxygen. Isometric tension changes were recorded. Dose-response measurements of carbachol-induced contractions, and a dose of carbachol (1·0–4·0 μg/ml) corresponding to about 80% of maximum contraction was then selected for use in the test of inhibitory activity of both drugs. These were administered at 0·008–0·8 μg/ml 90 s before carbachol. The decrease of carbachol-induced contraction was recorded. The dose of carbachol was then given every 8 min until a stable response again was obtained and the next β-adrenoceptor effect was evaluated.

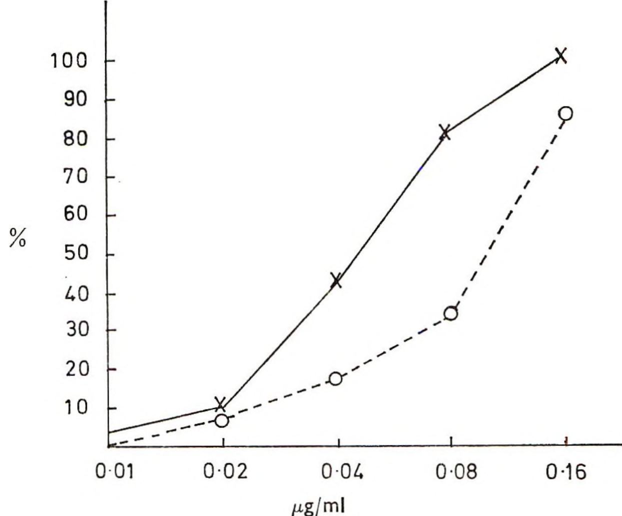

FIG. 1. The inhibition (%) of carbachol-induced contractions by different doses of terbutaline (×) and orciprenaline (○).

The effect of the β-adrenoceptor blocking agent propranolol (0·1 μg/ml) was determined by administering it to the bath 20 min before terbutaline or orciprenaline.

The amounts of drugs are expressed as the bases.

Twelve preparations from nine rats were used for the calculation of relaxing potency (ED50) of terbutaline and orciprenaline [terbutaline = 1·9 ($\pm$0·1 s.d.) $\times$ orciprenaline]. Slightly more than half the total number of preparations were discarded because of variation in the response to carbachol, or too much spontaneous activity, or because less than three dose-levels of the β-adrenoceptor stimulating compounds were evaluated.

Propranolol completely prevented the effect of both drugs given in doses that otherwise produced (60–80%) inhibition of the carbachol-induced contractions. A typical dose-response curve of the effects on the same preparation is shown in Fig. 1.

Terbutaline is seen to be about twice as potent as orciprenaline in relaxing the rat uterus. This ratio is similar to that found in the lung for the two compounds by Persson & Olsson (1970) who also reported orciprenaline to be more active than terbutaline on the heart (inotropic and chronotropic activity). The finding that terbutaline is more potent than orciprenaline on uterus is not surprising since Lands, Ludena & Buzzo (1967) have characterized the β-adrenoceptors in uterus to be of the same type (β_2-receptors) as in the lung and differing from the β-adrenoceptors in the heart (β_1-receptors).

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α-Methyltryptophan increases 5-hydroxytryptamine-like material in rat brain

Previous reports from this laboratory (Sourkes, Missala & Papeschi, 1969; Sourkes, Missala & Oravec, 1970) indicated that α-methyltryptophan (AMTP) induced a decrease of tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations in the brain of rats. Because AMTP had been found to increase tryptophan pyrrolase activity in the liver (Sourkes & Townsend, 1955), the hypothesis was put forward that the effect on brain 5-HT was mediated by an increased flow of tryptophan in the kynurenine pathway with consequent decreased availability of substrate for 5-HT synthesis. However, the decrease of the material estimated as 5-HT after administration of AMTP was not dose-dependent; AMTP itself may also be converted to α-methyl-5-HT (AM-5-HT) in the brain, as has been reported for α-methyl-5-hydroxytryptophan (Lahti & Platz, 1969). For these reasons we re-investigated the problem by using a different method to estimate 5-HT and 5-HIAA.

Previously 5-HT was estimated according to Snyder, Axelrod & Zweig (1965) (butanol extraction-ninhydrin condensation) and 5-HIAA by the method of Giacalone & Valzelli (1966) (butyl acetate extraction-3N HCl fluorescence). I have now compared the results obtained with these methods with results obtained by the method of Ahtee, Sharman & Vogt (1970), which is based on the sequential separation of 5-HT and 5-HIAA from the same sample by column chromatography on Amberlite CG-50, type I, and Sephadex G-10. The fluorescence in 3N HCl of both compounds was read in an Aminco-Bowman spectrophotofluorometer (excitation: 295 nm;

emission: 540 nm). Male, albino, Sprague-Dawley rats, of 150–200 g, were injected intraperitoneally with 50 mg/kg of DL-AMTP and killed 16 h later. The combined butanol–butyl acetate procedure showed a slight and not significant decrease of brain 5-HT concentration, with a marked and highly significant decrease of 5-HIAA. The latter finding was confirmed by the Amberlite-Sephadex method; in contrast, the material estimated as 5-HT in this method was significantly increased by over 50% (Table 1). A dose curve was then made by measuring 5-HT and 5-HIAA in the brain of rats 6 h after the injection of AMTP, by the Amberlite-Sephadex method. Whereas 5-HIAA showed a dose-dependent decrease, the "apparent" 5-HT was slightly decreased with the lowest, but increased with the highest AMTP doses (Table 2).

Table 1. *The effect of* DL-*AMTP on the concentration of 5-HT and 5-HIAA in the brain of the rat by different methods.*

Method	Treatment	No	Time (h)	5-HT* (ng/g)	*P***	5-HIAA* (ng/g)	*P***
(1) Butanol–butyl acetate extraction	Saline	4	16	584 ± 41	—	521 ± 31	—
	AMTP, 50 mg/kg	5	16	542 ± 8	NS	133 ± 6	<0·001
(2) Amberlite–Sephadex chromatography	Saline	12	16	431 ± 11	—	350 ± 49	—
	AMTP, 50 mg/kg	12	16	675 ± 41	<0·001	123 ± 18	<0·001

* = Mean ± s.e.
** = *t*-test, difference from controls.

Table 2. *Dose curve of the effect of AMTP on brain 5-HT and 5-HIAA, 6 h after the injection, by the Amberlite-Sephadex method.*

Treatment	No	5-HT* (ng/g)	*P***	5-HIAA* (ng/g)	*P***
Saline	4	423 ± 28	—	432 ± 33	—
AMTP, 12·5 mg/kg	4	370 ± 10	NS	239 ± 7	<0·005
AMTP, 25 mg/kg	4	380 ± 10	NS	273 ± 38	<0·02
AMTP, 50 mg/kg	4	571 ± 20	<0·01	196 ± 12	<0·001
AMTP, 100 mg/kg	4	650 ± 74	<0·05	177 ± 32	<0·005

* = Mean ± s.e.
** = *t*-test, difference from controls.

Table 3. *The effect of AMTP on 5-HT and 5-HIAA concentrations in the intestine of the rat and its interaction with yohimbine, by the Amberlite-Sephadex method.*

Treatment	No	Time (h)	5-HT* (μg/g)	*P***	5-HIAA* (ng/g)	*P***
Saline	4	—	5·314 ± 0·107	—	352 ± 26	—
AMTP, 50 mg/kg ..	4	16	4·413 ± 0·161	<0·01	302 ± 30	NS
Yohimbine, 5 mg/kg ..	3	2	6·475 ± 0·235	<0·01	317 ± 13	NS
Yohimbine 5 mg/kg + AMTP, 50 mg/kg ..	4	2 16	4·932 ± 0·194	NS	267 ± 18	<0·05

* = Mean ± s.e.
** = *t*-test, difference from controls.

The increase of 5-hydroxyindole material, estimated as 5-HT in the second method, cannot be accounted for by interference of AMTP itself; indeed, in our experimental conditions, pure solutions of AMTP yielded a fluorescence intensity that was 1/5000 that yielded by authentic 5-HT. Even supposing that AMTP is distributed evenly in the body and that the ratio of AMTP: 5-HT in the brain is in the order of 100 after injection of 50 mg/kg of AMTP, the interference of AMTP in the fluorescence readings could not have exceeded 2%. Thus, it appears that a compound is formed in the brain after injections of AMTP that is retained on the Amberlite columns and estimated as 5-HT, but that does not (or only slightly) interfere with the butanol-ninhydrin method. This compound is likely to be AM-5-HT which yields almost the same fluorescence intensity as 5-HT and has been isolated on paper chromatography from the brain of rats treated with AMTP (Roberge, Sourkes & Missala, in the press). On the other hand, the consistent decrease of brain 5-HIAA may stem from two possible effects of AMTP: (1) inhibition of monoamine oxidase, as the AM-5-HT formed would not be a substrate for this enzyme. (2) A decreased turnover of brain 5-HT due to the activation of liver pyrrolase; although the material estimated as 5-HT by the Amberlite-Sephadex method is increased, the "true" 5-HT may very well be decreased after AMTP.

The increase of 5-HT-like material occurs in the brain only; in fact AMTP (50 mg/kg, i.p.) decreased significantly 5-HT in the small intestine of the rat 16 h after the injection, and antagonized the increase of 5-HT induced by yohimbine (Papeschi, Sourkes & Youdim, 1971), as measured by the Amberlite-Sephadex method (Table 3).

Because of the possibility that AM-5-HT formed from AMTP acts as a false neurotransmitter, and because the synthesis of "true" 5-HT may be decreased after AMTP, I studied the effects of repeated injections of AMTP on the behaviour of the rat. Four animals were injected (100 mg/kg, i.p.) every 3 h (total: 400 mg/kg) on the first day; at 24, 27 and 30 h they received 100, 200 and 500 mg/kg of the drug, respectively. The behaviour was observed every hour for 5–10 min on both experimental days, up to 36 h after the first injection. Motor behaviour was also tested with the "vertical wire" and "four-corks" tests; rectal temperature was recorded with an electronic thermometer. No significant alteration of social or motor behaviour was observed at any time after AMTP, despite the marked changes in 5-HT metabolism that have been reported, and certainly no sedation was apparent. A slight but persistent hypothermia was instead observed (average 1° below the controls).

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Increased toxicity of morphine-like analgesics in aggregated mice

The early observations of an enhanced excitation (Gunn & Gurd, 1940) and increased lethality (Chance, 1946) after amphetamine in aggregated mice stimulated much subsequent investigation of the aggregation phenomenon. Thus, Greenblatt & Osterberg (1961) found that for aggregated mice the LD50 for some stimulants such as caffeine, picrotoxin or amiphenazole, and several phenethylamine derivatives, was significantly lower than that for isolated mice, but this was not so after phenelzine, mescaline, ephedrine, leptazol and bemegride. The stimulant activity of morphine in mice at the spinal cord level is well known from the Straub-tail phenomenon, and at the brain level by an enhancement of locomotor activity. The latter effect of morphine is antagonized by pretreatment with α-methyltyrosine (AMT), a finding that points to the morphine-induced activity being the result of a catecholamine-altering mechanism (Menon, Dandiya & Bapna, 1967). While Spoerlein (1968) has reported finding a difference in the acute LD50 of morphine for mice between aggregated and isolated conditions, Vedernikov (1970) found no difference in such a test. These considerations have led us to examine the influence of aggregation on the lethal dosage of several morphine-like analgesics and one morphine-like antagonist-analgesic, and to test combinations of AMT and the analgesics under similar conditions.

Swiss albino random-bred male mice of 26 to 32 g were housed for 7–10 days before experiments in plastic boxes (45 × 24 × 12·5 cm), 20–25 mice to a box. After being injected intraperitoneally they were placed in stainless steel cages (18 × 10 × 12·5 cm), either 1 or 5 to a cage. Three sides and the top were solid while the floor and front of the cage were of wire mesh. Preliminary results were the basis for selecting the 5 dosages used in each LD50 determination. Fifteen mice were treated at each dose level. Injections were begun at mid-morning and the mice were checked for deaths every half hour for the first 4 h and also at 12 and 24 h. Aggregated mice that died were removed and replaced by marked, untreated mice to maintain aggregation. Four h mortality figures were used to calculate LD50 values (Litchfield & Wilcoxon, 1949).

All test drugs significantly enhanced the acute lethality among aggregated animals, i.e. all had isolated: aggregated potency ratios significantly greater than one (Table 1). Although the ratios were statistically significant, several were so only by a slight

Table 1. *Effect of aggregation on lethality of mice treated with (+)-amphetamine or with analgesics.* Mice were aggregated in groups of 5 per cage. Fifteen mice were treated at 5 dose levels for each drug in both aggregated and isolated conditions. Figures in parentheses are 95% confidence limits.

Drug	Isolated LD50	Aggregated LD50	Potency ratio
(+)-Amphetamine sulphate	27·5 (25·1–30·1)	9·15 (8·36–10·0)	3·01 (2·68–3·37)
Levorphanol tartrate	146 (139–153)	106 (98·2–114)	1·38 (1·26–1·51)
Meperidine hydrochloride..	139 (125–155)	110 (98–124)	1·26 (1·07–1·49)
Methadone hydrochloride..	48·0 (45·7–50·4)	27·7 (25·4–30·2)	1·73 (1·58–1·90)
Morphine sulphate	470 (446–496)	413 (384–445)	1·14 (1·09–1·19)
Pentazocine lactate..	103 (97·9–109)	93·5 (90·0–97·0)	1·11 (1·06–1·15)

margin, pentazocine, morphine and meperidine having potency ratios of less than 1·30. On another occasion, however, we have observed a potency ratio for morphine as high as 1·62 (1·35–1·94). On the other hand, the values found for levorphanol and methadone were 1·38 and 1·73, respectively. All of these potency ratios are significantly less than that for (+)-amphetamine, 3·01. In no comparison was there a significant difference in the slopes of the regression lines for aggregated and isolated lethality.

Whether the enhancement of toxicity by aggregation is dependent upon a release of brain catecholamines, was challenged by testing the ability of (±)-α-methyl-*p*-tyrosine (AMT) to counteract the aggregation effect for all five analgesics and for (+)-amphetamine. Methods were as described above except that only a single dose level of the test drugs was used, the incidence of mortality of saline plus test drug to AMT + test drug being compared by the χ^2 test (Siegel, 1956). Two doses of AMT, 50 mg/kg each, were administered as a suspension in saline at 12 and 4 h before injection of the test drug.

No reduction of lethal toxicity of the analgesics was produced by AMT, although the dosage schedule was effective in protecting mice treated with (+)-amphetamine. Protection against amphetamine-induced lethality was highly significant even with only a single 50 mg/kg dose of AMT at the 4 h pretreatment time (10/25 deaths for AMT–amphetamine and 21/24 deaths after saline–amphetamine). The 2-dose AMT schedule was found to have less effect on lethality of (+)-amphetamine in isolated mice (3/25 *vs* 6/25 deaths 4 h after 35 mg/kg of (+)-amphetamine, and 4/25 *vs* 10/25 at 10 h). These results are in accord with the report of Spoerlein (1968) in which she stated that several catecholamine-modifying agents failed to antagonize the lethality of morphine in grouped mice. Agents she tried were reserpine, chlorpromazine, α-methyl-*m*-tyrosine, α-methyldopa, tolazoline, phenoxybenzamine, propranolol, tyramine, guanethidine, imipramine and phenelzine. As expected, the results of the AMT-amphetamine combination are in accord with results reported previously on this interaction (Mennear & Rudzik, 1966; Menon & Dandiya, 1967).

To test for the possible involvement of 5-hydroxytryptamine in the aggregation lethality phenomenon of the analgesics, *p*-chlorophenylalanine (PCPA) pretreatment was given before test doses of morphine and meperidine. The dosage schedule of 100 mg/kg of PCPA repeated 3 times at 72, 48 and 24 h before the analgesics gave no sign of altering the aggregated lethality of analgesics. This result is of interest in view of the recent report of Jounela (1970) that PCPA pretreatment was able to antagonize the combined lethality of phenelzine and meperidine in aggregated mice. However, he did not test whether such pretreatment could modify toxicity of meperidine alone.

Whereas Jounela's results indicate that a 5-HT system is involved in the synergistic interaction of MAO inhibitors and narcotic analgesics, it does not appear that such a system is relevant to the aggregated lethality of mice with such analgesics alone. Our AMT–morphine evidence also appears to exclude any role of a catecholamine-related mechanism in this aggregated lethality response, a circumstance by which the response can be clearly differentiated from the aggregation lethality of (+)-amphetamine.

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A possible synaptic mechanism underlying the similar behavioural effects of adrenaline-like and acetylcholine-like drugs

There are many situations in which adrenaline-like and acetylcholine-like compounds produce similar effects. Amphetamine, a drug that increases activity at adrenoceptive synapses by preventing the reuptake of released noradrenaline (Rutledge, 1970), has behavioural effects very similar to those produced by the two closely related muscarinic blockers hyoscine and atropine. Both amphetamine and atropine disrupt timing behaviour on Fixed Interval (Ray & Bivens, 1968) and on differential reinforcement of low rates of response (Carlton, 1963; Bivens & Ray, 1968) operant conditioning schedules; both increase response rates on a Sidman avoidance schedule (Carlton, 1963; Ray & Bivens, 1968); and both increase responding during periods of non-reward in a discrete trial bar press task (Heise, Laughlin & Keller, 1970). A behavioural tolerance is quickly formed to chronic doses of both amphetamine (Schuster & Zimmerman, 1961) and hyoscine (Bignami & Gatti, 1968) in situations where the initial drug effect is a reduction in reinforcement. This suggests that the same behavioural effects produced by adrenergic stimulation and cholinergic blockade could arise from a common neural mechanism rather than necessarily from two complimentary neural systems.

The existence of a common mechanism is also suggested by the demonstration that subthreshold doses of atropine and amphetamine, when given simultaneously, act additively to give the same effect as do larger doses of either drug given alone (Carlton, 1963; Bivens & Ray, 1968; Ray & Bivens, 1968). Although these facts might be reconciled by postulating reciprocally acting adrenergic and cholinergic systems with adrenergic stimulation having the same behavioural effect as cholinergic blockade, the demonstration that adrenergic activation also produces the same effect as cholinergic activation does not fit the reciprocally acting systems hypothesis. Intracranial self-stimulation of the lateral hypothalamus is depressed by adrenaline (Mogenson, Russek & Stevenson, 1969), by noradrenaline (Olds, Yuwiler & others, 1964), by amphetamine (Umemoto & Kido, 1967) as well as by the centrally-active cholinergic agonist physostigmine (Domino & Olds, 1968; Stark, Totty & others, 1968; Olds & Domino, 1969a, 1969b). The lateral hypothalamus in turn makes adrenergic inhibitory synapses in the amygdala (Stein & Wise, 1969) and cholinergic inhibitory

synapses in the cortex (Phillis & York, 1968), but the behavioural significance of these output systems of the lateral hypothalamus has not yet been elucidated.

As suggested by the two types of inhibitory synapses made by the lateral hypothalamus, noradrenaline and acetylcholine also produce the same electroencephalographic responses. Cortical e.e.g. arousal is produced by both adrenaline (White & Daigneault, 1959) and acetylcholine (Domino, Dren & Yamamoto, 1967). Both neurotransmitters cause excitation of the caudate putamen region (York, 1968) and of some Renshaw cells in the spinal cord (Weight & Salmonoiraghi, 1966), although Renshaw cells are more typically excited by acetylcholine and depressed by noradrenaline (Salmoiraghi, 1966). A further interrelation of adrenaline-like and acetylcholine-like neurotransmitters is seen by the demonstration that hyoscine blocks the e.e.g. activation produced by amphetamine or adrenaline (White & Daigneault, 1959) or by acetylcholine (Domino & others, 1967). Conversely, Phillis (1970) reported that phentolamine blocks the cortical depressant effects of acetylcholine as well as the depressant effects of noradrenaline. Thus the neurotransmitter effects of synaptic blockade need not be limited to the neurotransmitter system being blocked.

In certain peripheral organs, acetylcholine activity seems to be very closely related to noradrenaline activity too. Increasing cholinergic activity by blocking the deactivation of acetylcholine with injections of physostigmine produces an adrenergic hypertensive response (Varagić & Krstić, 1966). The synaptic mechanism involved may be deduced from evidence (Belej, Papacostas & others, 1968; Muscholl, 1970) demonstrating a nicotinic acetylcholine-induced release of noradrenaline in isolated heart tissue that is inhibited after several seconds by a muscarinic acetylcholine action (Muscholl, 1970). The acetylcholine-induced release of noradrenaline, first demonstrated in the sympathetic nervous system by Burn & Rand (1959, 1962), has been observed recently in thalamocortical recruitment (Karczmar, 1969) and in the hypothalamus (Philippu, Heyd & Burger, 1970). The hypothesis that acetylcholine may be involved in the release of noradrenaline into the synapse following a nerve impulse received histological support by the demonstration of acetylcholinesterases and catecholamine (typically noradrenaline) in the same nerve trunk (Jacobowitz, 1965; Jacobowitz & Koelle, 1965; Eranko, 1966). Philippu & others (1970) noted that the presence of calcium ions was necessary for the occurrence of acetylcholine-induced release of noradrenaline. The mechanisms presented by Karczmar (1969) and Philippu (1970) account for the role of calcium in this system. That an analogous acetylcholine-5-hydroxytryptamine relation does not exist is suggested by the data presented by Katz & Kopin (1969) that demonstrated that 5-HT release is not calcium dependent.

The hypothesis of acetylcholine-mediated release of noradrenaline accounts for all of the evidence presented above showing identical effects of cholinergic and adrenergic stimulation. Philippu (1970) also reported that the acetylcholine-induced release of noradrenaline is increased by atropine, a finding that would be expected since Muscholl (1970) demonstrated that the nicotinic release of noradrenaline in the heart is blocked by muscarinic activity. Therefore the similar behavioural effects of amphetamine and hyoscine discussed above are also accommodated by this hypothesis. Amphetamine increases adrenergic activity by blocking reuptake of noradrenaline while hyoscine increases adrenergic activity by preventing the muscarinic inhibition of the acetylcholine-induced release of noradrenaline. The net effect of both drugs is an increase of noradrenaline in the synapse. Although both Koelle (1969) and Karczmar (1969) confirm the existence of a pure cholinergic synapse, i.e. one in which only acetylcholine participates in neurotransmission, the histological and behavioural separation of dual and pure cholinergic synapses remains to be accomplished.

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The effect of pethidine on the 5-hydroxytryptamine and 5-hydroxyindoleacetic acid content of the mouse brain

Morphine decreases hypothalamic noradrenaline in cats (Vogt, 1954) and dopamine in mouse brain (Takagi & Nakama, 1966). Chronically administered morphine is reported to increase the turnover of 5-hydroxytryptamine (5-HT) in the brain of rats and mice (Shen, Loh & Way, 1970; Haubrich & Blake, 1969; Bowers & Kleber, 1971) although not in all experiments (Marshall & Grahame-Smith, 1970; Algeri & Costa, 1971; Cheney, Goldstein & others, 1971). Bowers & Kleber (1971) showed that methadone increases mouse brain 5-hydroxyindoleacetic acid (5-HIAA) content both in acute and chronic experiments. However, in heroin addicts treated with methadone, probenecid caused less increase in the 5-HIAA content of cerebrospinal fluid than in control patients (Tamarkin, Goodwin & Axelrod, 1970).

We now report the effect of pethidine on the 5-HT and 5-HIAA content of the mouse brain *in vivo*. Probenecid blocks the active transport of 5-HIAA in rat and mouse brain (Neff, Tozer & Brodie, 1967; Sharman, 1969). Chronic treatment with lithium interferes with the monoamine uptake by synaptosomes and platelets (Colburn, Goodwin & others, 1967; Murphy, Colburn & others, 1969).

White male mice, 20–25 g, were kept in groups of eight; experiments were made between 4 and 8 p.m., at 25°. Drugs were injected in 0·1 ml/10 g and controls injected with similar amounts of saline. The animals were decapitated always between 7 and 8 p.m. and the brains, except cerebellum, were rapidly removed, weighed and stored at $-20°$ for less than 5 days. 5-HT and 5-HIAA were estimated in the same tissue sample spectrophotofluorimetrically (Ahtee, Sharman & Vogt, 1970). Pethidine did not interfere with the fluorescence of 5-HT or 5-HIAA. Pethidine hydrochloride was dissolved in saline. Probenecid (Benemid; a gift from Merck Sharp & Dohme Ltd) was dissolved in a minimum volume of 1N NaOH and the pH of the solution was adjusted to 7–8 with 0·1N HCl and diluted further with saline. The doses are expressed as base. In one series of experiments mice were pretreated with lithium chloride administered in drinking water (200 mg LiCl in 1000 ml of tap water) for three months.

Pethidine alone (80 mg/kg, i.p., 2 h) did not alter the concentration of brain 5-HT or 5-HIAA (Table 1). In preliminary experiments, 30, 60 and 120 mg/kg of pethidine were given intraperitoneally to the mice. No changes in brain 5-HT or 5-HIAA content were observed 2 h after the lower doses and 30 min after the highest dose. In our mouse strain the LD50 of pethidine is 113 mg/kg, i.p. (Jounela, Saarnivaara & Ahtee, 1971).

Probenecid (200 mg/kg, i.p., $1\frac{3}{4}$ h) increased the 5-HIAA content in the brain of the saline- and pethidine-treated mice. But, the increase in the brain 5-HIAA content was statistically significantly higher in the control group ($P < 0{\cdot}001$ by *t*-test after pairing values). The time course of the effect of combined pethidine-probenecid treatment on the 5-HIAA content was also studied by giving probenecid

Table 1. *Effect of pethidine* (80 mg/kg, i.p., 2 h) *and probenecid* (200 mg/kg, i.p., $1\frac{3}{4}$ h) *on the 5-HT and 5-HIAA content of mouse brain.* Means ± s.e.; number of mice in brackets.

Treatment	5-HT μg/g	5-HIAA μg/g
Saline	0·577 ± 0·033 (8)	0·367 ± 0·022 (7)
Probenecid	0·609 ± 0·033 (10)	0·648 ± 0·062 (7)
Pethidine	0·619 ± 0·017 (7)	0·343 ± 0·028 (7)
Pethidine + probenecid ..	0·623 ± 0·017 (9)	0·494 ± 0·020 (7)

$1\frac{3}{4}$ h before killing and varying the duration of pethidine treatment. The lowest 5-HIAA content was found 2 h after administration of pethidine; 3 h after pethidine the 5-HIAA values were 0·556 and 0·563 μg/g and 4 h after pethidine 0·589 $\pm$ 0·066 μg/g (mean $\pm$ s.e.; n = 4). Lithium pretreatment neither altered the brain 5-HT and 5-HIAA contents nor modified the effects of pethidine or probenecid. In the lithium-pretreated mice the increase in the brain 5-HIAA content was twice as much after probenecid only, as after the combination of pethidine and probenecid.

Fifteen min after injection of pethidine (80 mg/kg, i.p.) the mice became excited, jumpy, had motor disturbances and exhibited the Straub tail phenomenon. These symptoms wore off in about 2 h. At this time the mice were sedated. Probenecid or lithium did not modify these symptoms. No, or only slight, decrease of rectal temperature was caused by pethidine or probenecid at the temperature of 25°.

Thus, pethidine decreases the probenecid-induced accumulation of 5-HIAA in mouse brain, an effect which could arise from the inhibition of the re-uptake of 5-HT into neurons, whereby 5-HT would not reach intraneuronal monoamine oxidase (MAO). This possibility is supported by the finding that at 50 mg/kg, i.p., pethidine prevents the 4-methyl-α-ethyl-*m*-tyramine (H 75/12)-induced 5-HT displacement in the mouse brain *in vivo* (Carlsson & Lindqvist, 1969) as well as by our experiments in which pethidine prevented the uptake of 5-HT into human blood platelets *in vitro* (Ahtee & Saarnivaara, 1970a, b). Furthermore, Meek & Werdinius (1970) have shown that chlorimipramine, a potent inhibitor of 5-HT uptake, decreases the probenecid-induced accumulation of 5-HIAA in rat brain. But there are several other possible causes for the effect of pethidine—such as the inhibition of 5-HT oxidation by MAO, decrease in the synthesis or release of 5-HT, and pethidine might in one way or another antagonize the blockade of active transport caused by probenecid.

At least some actions of pethidine could be mediated by alterations of the brain 5-HT content. Recent evidence shows that the toxic reactions of pethidine when combined with MAO inhibitors are at least partially due to increased 5-HT concentrations in the mouse brain (Jounela, 1970; Rogers & Thornton, 1969; Rogers, 1971). Moreover, 5-HT is involved in the perception of pain and in the mechanism of action of several analgesics (Tenen, 1967 & 1968).

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Comparison of four vehicles for intraperitoneal administration of Δ^1-tetrahydrocannabinol*

Recently there has been much interest in research on Δ^1-tetrahydrocannabinol, commonly designated by the abbreviation Δ^1-THC (or Δ^9-THC), which is believed to be the principal active constituent of marihuana. However, a practical difficulty of research with Δ^1-THC is its insolubility in water and many other common solvents. It is soluble in ethanol, but this vehicle is pharmacologically active, in particular resembling effects of Δ^1-THC in general depressant action (Kubena & Barry, 1970) and in stimulation of the adrenal-pituitary system (Kubena, Perhach & Barry, 1971). Another solvent for Δ^1-THC, propylene glycol, was used by Bose, Saifi & Bhagwat (1964) and by Bicher & Mechoulam (1968), but this is also a general depressant when given in large amounts (Bost & Ruckebusch, 1962). Therefore, in the studies cited and others (Sofia & Barry, 1970; Sofia, Dixit & Barry, 1971), the present authors used a suspension in 10% propylene glycol, 1% polysorbate (Tween) 80 and isotonic saline to minimize the amount of propylene glycol administered.

Fenimore & Loy (1971) have recently suggested the use of a suspension of Δ^1-THC in polyvinylpyrrolidone (PVP), a plasma expander. Other vehicles used for intraperitoneal administration of Δ^1-THC include a suspension in bovine serum albumin (Dewey, Peng & Harris, 1970; McMillan, Harris & others, 1970) and a suspension in Tween 80-saline (Holtzman, Lovell & others, 1969). Recently, Ho, Fritchie & others (1971) presented evidence for poor absorption of Δ^1-THC after intraperitoneal injection in a Tween-80-saline suspension. They recommended that the drug be administered intravenously, but this route has the drawback of being more difficult and stressful, especially for rats, and not directly comparable with data on other drugs, which are mostly administered intraperitoneally.

The present report compares four vehicles in efficacy and duration of effect after intraperitoneal injection of Δ^1-THC. The latency to convulsion in mice following a maximal electroconvulsive shock (ECS) was used as a bioassay of the Δ^1-THC effect. Recent work (Sofia, Solomon & Barry, 1971) has shown this to be a sensitive measure of Δ^1-THC, even at a low dose, when injected intraperitoneally in a 10% propylene glycol–1% Tween 80-saline suspension.

The experiment was made on 384 male albino mice (Swiss-Webster), 20 to 22 g (Hilltop Lab Animals, Inc., Scottdale, Pa.). They were divided randomly among the

*Numbered Δ^9 according to IUPAC rules.

four vehicle conditions: bovine serum albumin-saline (BSA), 1% Tween 80-saline (Saline), polyvinylpyrrolidone-saline (PVP), and 10% propylene glycol-1% Tween 80-saline (PG). For all the vehicles the volume of fluid injected was 0·1 ml/10 g (10 ml/kg), containing Δ^1-THC in a concentration of 1 or 4 mg/ml depending on the dosage (10 or 40 mg/kg).

The BSA was prepared in a solution of 50 mg/ml in isotonic saline. The Saline vehicle was also isotonic and included a 1% suspension of Tween 80. The Δ^1-THC, received from the FDA-NIMH Psychotomimetic Drugs Advisory Committee in a 99% ethanol solution (100 mg/ml), was flash evaporated and within 10 min added to the vehicle. For the PG vehicle the flash-evaporated Δ^1-THC was dissolved in 100% propylene glycol (10 or 40 mg/ml), to which the other fluids were immediately added to make up a suspension of Δ^1-THC in 1% Tween 80 and 10% propylene glycol in isotonic saline. For the PVP vehicle, the original ethanol solution of Δ^1-THC (10 or 40 mg) was added to 300 mg of PVP in a 95% ethanol solution (100 mg/ml). The ethanol was flash evaporated and sufficient isotonic saline was added to make up a suspension containing 30 mg/ml of PVP.

These four vehicle groups were subdivided into groups tested at four time intervals after intraperitoneal injection (0·25, 1, 2, 6 h). Half the animals were injected with the vehicle alone (control), half with Δ^1-THC suspended in the vehicle. Half the animals, tested on one day, were equally divided between control and the lower dose of Δ^1-THC (10 mg/kg). The other half were divided between control and the higher dose of Δ^1-THC (40 mg/kg). Each of the animals tested on the same day were housed in the laboratory in groups of 12 for 24 h before use and were divided at the time of injection into 6 vehicle and 6 drug animals. The mice were tested for latency to tonic convulsion, i.e. complete extension of the hind limbs, on electroshock of 50 mA intensity, 0·2 s duration, applied transcorneally. Latency to tonic extension was recorded by stopwatch in 0·1 s units.

Table 1. *Effects of Δ^1-THC on latency to convulsion (mean ± s.e.) at four time intervals after intraperitoneal injection in four vehicles.**†

Vehicle	Time (h)	Control N = 12	Δ^1-THC 10 mg/kg N = 6	Δ^1-THC 40 mg/kg N = 6	% Difference from control 10 mg/kg	% Difference from control 40 mg/kg
BSA	0·25	1·50 ± ·08	1·60 ± ·21	1·77 ± ·11	7	18
	1	1·52 ± ·11	1·62 ± ·20	1·95 ± ·21	7	28
	2	1·50 ± ·08	1·28 ± ·07	1·53 ± ·10	—15	2
	6	1·32 ± ·08	1·35 ± ·05	1·53 ± ·12	2	16
Saline	0·25	1·30 ± ·04	1·48 ± ·16	1·65 ± ·13	14	27*
	1	1·46 ± ·09	1·42 ± ·12	1·70 ± ·07	—3	16
	2	1·69 ± ·11	1·48 ± ·11	1·53 ± ·11	—12	—9
	6	1·48 ± ·11	1·63 ± ·21	1·55 ± ·07	10	5
PVP	0·25	1·47 ± ·08	1·97 ± ·16	2·17 ± ·17	34*	48**
	1	1·53 ± ·09	1·78 ± ·05	3·77 ± ·52	16	146**
	2	1·46 ± ·06	1·65 ± ·10	2·92 ± ·40	13	100
	6	1·38 ± ·06	1·50 ± ·07	1·50 ± ·09	9	9
PG	0·25	1·54 ± ·08	2·32 ± ·20	3·63 ± ·36	51**	136**
	1	1·53 ± ·05	2·03 ± ·20	4·83 ± ·17	33*	216**
	2	1·48 ± ·08	2·07 ± ·16	4·57 ± ·41	40*	209**
	6	1·48 ± ·06	1·75 ± ·14	2·13 ± ·25	18	44*

† A maximum interval of 5 s was recorded for animals which failed to convulse by that time (2 on PVP at 1 h, 5 on PG at 1 and 4 on PG at 2 h, all after 40 mg/kg Δ^1-THC).
* $P < 0·01$ for difference from control.
** $P < 0·001$ for difference from control.
BSA = bovine serum albumin. Saline = 1% Tween 80-saline. PVP = polyvinylpyrrolidone-saline. PG = 10% propylene glycol 1% Tween 80-saline.

Table 1 summarizes the results of the experiment. The control animals tested on different days, for comparison with different doses of Δ^1-THC, showed no statistically significant differences from each other and are combined into a single group of 12 animals under each experimental condition. There was no reliable difference among the four vehicles without the drug; the last two columns of the Table show that Δ^1-THC generally increased latency of the ECS response, with a much larger effect of the higher dose and with differences among the vehicles.

In each of the eight conditions comprising both dose levels and all four time intervals, the percentage increase in latency was larger for the PG than any of the other three vehicles. The PVP was the second most effective vehicle, and at the first three time intervals a larger increase was invariably found with PVP than with either of the other two vehicles. The peak effect for both PG and PVP was at 0·25 h with 10 mg/kg and at 1 h with 40 mg/kg, indicating a somewhat earlier time of peak action with the lower dose. The other vehicles (BSA, Saline) showed very little difference from control; the only reliable difference was with the high Δ^1-THC dose at 0·25 h with the Saline vehicle.

The failure of Δ^1-THC absorption from the peritoneal cavity, reported by Ho & others (1971) with the saline vehicle, is confirmed and extended to the BSA vehicle in the present study. Observations indicated that both of these vehicles failed to form satisfactory Δ^1-THC suspensions. The PVP forms an effective suspension but being a larger molecule than PG might not be absorbed readily from the peritoneal cavity. The PG vehicle was also observed to form an effective suspension, and the present data indicate that it is the vehicle of choice for intraperitoneal administration of Δ^1-THC.

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The centrally induced fall in blood pressure after the infusion of amphetamine and related drugs into the vertebral artery of the cat

According to Schmitt, Schmitt & Fenard (1971) the central hypotensive action of clonidine is due to the stimulation of central α-adrenoceptors belonging to an inhibitory neuron, located in the region of the nucleus tractus solitarii. The stimulation of such receptors would lead to a *decrease* in sympathetic tone in the periphery, thus bringing about a reduction in blood pressure similar to that induced by short-lasting stimulation of the carotid sinus. According to this view, the antihypertensive drug clonidine should be considered as a *sympathomimetic* agent with predominating central action, although weak peripheral sympathomimetic properties have also been demonstrated (Hoefke & Kobinger, 1966). If this hypothesis is correct, all sympathomimetic agents with central action should decrease peripheral sympathetic tone when applied to the region where the postulated α-receptors are located. For this purpose we studied the influence of amphetamine and some related compounds on blood pressure after infusion into the left vertebral artery. Upon infusion into the cat left vertebral artery the test drug will initially reach the rhombencephalon where the receptors are believed to be located. The technique has been described (van Zwieten, Bernheimer & Hornykiewicz, 1966; Sattler & van Zwieten, 1967; Henning & van Zwieten, 1968). The experiments were made in cats of either sex (2–4 kg) anaesthetized with 60 mg chloralose/kg, given intraperitoneally. The drugs (in saline) were infused into the left vertebral artery at a rate of 0·1 ml kg^{-1} min^{-1}. Arterial blood pressure was taken from a femoral artery and recorded continuously on a smoked drum. Amphetamine (50 μg/kg) caused an acute and pronounced fall in blood pressure (∼20 mm Hg), although the intravenous injection of the same dose increased pressure by about 30% of its control value. A higher dose (150 μg/kg) of amphetamine decreased blood pressure by approximately 35% of its control value after infusion into the vertebral artery. The effects were confirmed in at least 6 cats for each dose. Intravenously injected piperoxan (0·6 mg/kg) caused a transient fall in blood pressure by about 10 mm Hg. After normalization of pressure the infusion of amphetamine (150 μg/kg) into the vertebral artery did not influence blood pressure any more (n = 4). Similar results were obtained with yohimbine (n = 3). Consequently, both α-sympatholytic agents abolish the central hypotensive action of amphetamine (present studies) but also that of clonidine (Schmitt & others, 1971). Similarly, pretreatment of the animals with 1 mg haloperidol/kg, given intravenously 30 min before amphetamine (150 μg/kg, vertebral artery) abolished the central hypotensive action of the latter drug. Haloperidol has been reported to block central adrenoceptors (Andén, Corrodi & others, 1970). In reserpine-pretreated cats (1 mg/kg, 24 h before the actual experiment), the blood pressure was about 20 mm lower than in normal cats. In reserpinized cats the infusion of amphetamine 150 μg/kg into the left vertebral artery did not reduce blood pressure, but induced a small hypertensive effect probably due to the transition of amphetamine into the peripheral circulation. The present studies suggest that, like clonidine, amphetamine is also capable of stimulating the hypothetical α-adrenoceptors in the rhombencephalon, resulting in a decrease in peripheral sympathetic tone. The stimulation of the α-receptors in the cns after infusion of amphetamine into the vertebral artery probably occurs by noradrenaline, mobilized by amphetamine rather than by the drug itself, since the central hypotensive action did not occur in reserpinized rats. The injection of noradrenaline into the cisterna or into the lateral ventricle (to avoid the blood brain barrier) have been reported to decrease peripheral sympathetic tone

(Kaneko, 1960; McCubbin, 1960). Clonidine, which does not mobilize noradrenaline in the brain, probably stimulates the α-receptors itself (direct central sympathomimetic action).

Ephedrine (50 μg/kg), phentermine (300 μg/kg), and chlorphentermine (300 μg/kg) also reduced blood pressure after infusion into the left vertebral artery. The effect of ephedrine developed rather slowly and persisted for about 1 h. Chlorphentermine was more active than phentermine. Intravenously injected ephedrine (50 μg/kg) or phentermine (300 μg/kg) showed hypertensive effects, but chlorphentermine slowly reduced blood pressure after intravenous injection (300 μg/kg). Probably, the central effect of this drug is quantitatively more important than its peripheral sympathomimetic properties. The high lipid solubility of chlorphentermine may contribute to this phenomenon.

The decrease in peripheral sympathetic tone, initiated by the stimulation of central α-adrenoceptors seems to be a general principle which would apply to all drugs with central sympathomimetic properties when applied via a central route of administration. Until now, clonidine is the most potent drug in this context. The central hypotensive properties of α-methyldopa (Henning & van Zwieten, 1968), L-dopa (Rubenson, 1971) and *m*-tyrosine (Rubenson, 1971) might also be explained by such a mechanism, since in the brain these amino-acids are known to be converted into sympathomimetic agents that may stimulate the central α-adrenoceptors.

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The effect of diethyldithiocarbamate on amphetamine-induced behaviour in rats

Sodium diethyldithiocarbamate is a potent inhibitor of the enzyme dopamine-β-hydroxylase of brain *in vivo* (Carlsson, Fuxe & Hökfelt, 1967; Lippmann & Lloyd, 1969). It causes a depression of the conditioned-avoidance responses (Krantz & Seiden, 1968), but an excitation of agressive traits when combined with pargyline (Scheel-Krüger & Randrup, 1968). We report the effect of diethyldithiocarbamate on two aspects of amphetamine-induced-hyperactivity in rats.

Female albino rats (Wistar), 180–210 g, of the Lysolaje strain, were tested.

Sodium diethyldithiocarbamate (DDC, Lachema, Brno), and commercial solutions of amphetamine sulphate (AMPH, Psychoton) and imipramine (Melipramin, Vereinigte Heil and Nährmittelwerke Budapest) were administered intraperitoneally diluted in water at doses of DDC 400 and 500 mg/kg, AMPH 4 and 5 mg/kg, and imipramine 3 mg/kg. Leptazol (pentetrazolium; PhBS III) was dissolved in saline solution (0·5%), and administered by intravenous infusion.

The horizontal component of exploratory activity of the rats (number of crossed squares) was automatically recorded (Žalud, Mysliveček & others, 1970) in a darkened room at 23 $\pm$ 0·5° for 3 min. One day before, and on the same day as the test the animals were placed for 1 h in the room but not in the experimental box). The first measure of the parameter was made 60 min after administration of DDC or 30 min after AMPH, and the second measure 120 min after DDC or 90 min after AMPH.

The stereotyped movements provoked by amphetamine were seen in rats given 5 mg/kg. These were defined (Lapin & Shchelkunov, 1963) as a characteristic immobile posture with stereotyped movements of the head and front limbs; the numbers of rats making these movements were recorded at regular intervals.

In untreated rats, the horizontal component value for exploratory activity (Fig. 1) was 30 crossed squares in the first, and about 15 in the second measurement. DDC, 500 mg/kg, decreased the activity; this effect is more conspicuous at the first measurement. AMPH, 4 mg/kg, increased the activity, the corresponding values being twice as high as in the controls ($P < 0·01$) at the first measurement, whereas at the second, there appeared no significant differences in the values.

When combined, the drugs (DDC administered 30 min before AMPH) gave a lower exploratory activity than in the AMPH group, but still higher than in the control group ($P < 0·05$).

Table 1. *Duration of stereotyped movements provoked by amphetamine in rats pretreated with diethyldithiocarbamate or imipramine.* The numbers of rats producing the sterotyped movements are given. Diethyldithiocarbamate (400 mg/kg) and imipramine (3 mg/kg) were administered i.p. 30 min before amphetamine (5 mg/kg).

	No. of rats per group	Time (min) 30	40	50	60	75	90	105	120	135	150	165	180	210	240
Amphetamine control ..	7	0	2	2	3	6	7	7	7	4	3	2	2	0	—
Diethyldithiocarbamate + amphetamine	7	3	3	7	7	7	7	7	7	7	7	5	5	4	—
P				<0·01	<0·05						<0·05			<0·05	
Amphetamine control ..	7	2	2	6	6	6	6	6	6	4	3	2	1	0	0
Imipramine + amphetamine ..	6	1	1	3	5	6	6	6	6	6	6	6	6	6	6
P				<0·05									<0·05	<0·01	<0·01

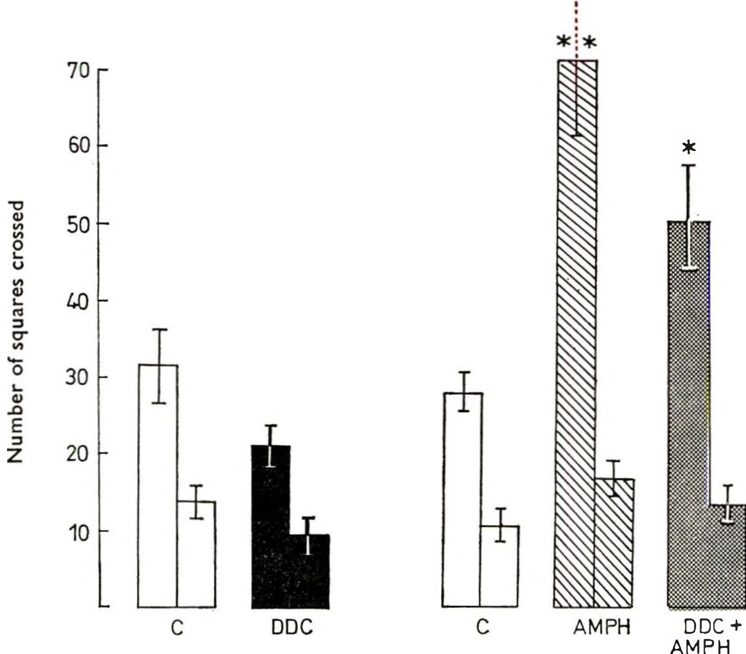

FIG. 1. Horizontal component of exploratory activity. Rats received i.p. distilled water (controls), diethyldithiocarbamate (DDC) 500 mg/kg, amphetamine (AMPH) 4 mg/kg and combination of DDC and AMPH respectively. Each pair of columns represent the first and second measurement. The mean value ± s.e. and the significance of differences (against the time corresponding control group) calculated by *t*-test are given, $^{**}P < 0{\cdot}01$, $^{*}P < 0{\cdot}05$.

Amphetamine stereotyped movements were perceptible (see Table 1) in most of the treated rats during 90 to 120 min after the AMPH, 5 mg/kg. DDC pretreatment, 30 min before AMPH, produced an earlier start, and a longer duration of these movements ($P < 0{\cdot}01$); and they were seen in 4 rats in the DDC-pretreated group, but in none of the non-pretreated group at 210 min after AMPH.

Imipramine pretreatment, 30 min before AMPH, delayed the start of the movements ($P < 0{\cdot}05$) but prolonged their duration beyond the limit of 240 min; all rats of the imipramine group, but none of the non-pretreated group, showed the movements 210 and 240 min after AMPH.

DDC possesses neither an antitremorine (Pfeifer, Galambos & György, 1966) nor an anticonvulsive action (since we have found that the amount of leptazol intravenously infused into mice to give convulsions remained unchanged after DDC, 500 mg/kg given 1 h before the infusion) but prolongs hexobarbitone sleep (Lange, Kästner & Jung, 1970; Pfeifer, Galambos & György, 1966). This is seen indirectly in that the administration of doses up to 500 mg/kg, as used in most experiments (ours; those of Pfeifer, Galambos & György, 1966; Krantz & Seiden, 1968; Scheel-Krüger & Randrup, 1968; Przegalinski & Kleinrok, 1970), is unlikely to provoke a non-specific cns depression of a toxic character. The hexobarbitone-potentiating effect is rather to be attributed to an inhibition of the liver microsomal oxidation by DDC (Lange & others, 1970).

The DDC effects on animal behaviour may be explained as an alteration of the dopamine-β-hydroxylase activity in the brain. The noradrenaline content is decreased after a single administration of DDC (Przegalinski & Kleinrok, 1970).

The dopamine content rises after repeated administration of DDC (Scheel-Krüger, & Randrup, 1968) or when given directly into the lateral ventricle of the brain (Kleinrok, Żebrowska & Wielosz, 1970). The changes are the result of dopamine-β-hydroxylase inhibition. Moreover, an increase of the tyrosine content in the brain probably caused by the feedback mechanism has been described (Magos & Jarvis, 1970). We found DDC to depress exploratory activity both in untreated and AMPH-stimulated rats on the one hand, while on the other it prolonged the duration of amphetamine stereotyped behaviour. Analogous results have been obtained by D'Encarnacao, D'Encarnacao & Tapp (1969) with AMPH-stimulated animals. These experiments support the hypothesis that large locomotory movements (AMPH-hypermotility) are provoked by release of noradrenaline, while the small movements, of stereotypic character, result from dopamine release (D'Encarnacao & others, 1969; Randrup & Scheel-Krüger, 1966). The DDC effects we found may be similarly explained in terms of the decrease of noradrenaline content (with a decrease of exploratory activity), and at same time, a dopamine rise (with a prolongation of amphetamine stereotyped behaviour).

DDC is considered to increase lethality in aggregated mice (Przegalinski & Kleinrok, 1970) and combined with pargyline provokes agressiveness in rats that is accompanied by a decrease in brain noradrenaline and a rise in dopamine (Scheel-Krüger & Randrup, 1968).

It seems that the elevated dopamine concentration in the brain should play a specific role in the phenomenon of adrenergic excitation described by Lapin & Shchelkunov (1963); hence it may be noted that the antidepressants like imipramine prolong the duration of amphetamine stereotypes and potentiate the toxicity of AMPH in aggregated mice (Simon, 1965).

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Changes in brain monoamine concentrations during the oestrous cycle in the mouse: possible pharmacological implications

Concentrations of noradrenaline in the hypothalamus change during periods in which hormone levels are fluctuating (Stefano & Donoso, 1967; Green & Miller, 1966) but information on changes in the concentrations of the biogenic amines in other areas of the brain, or on changes occurring at specified times during the oestrous cycle is scant. Because of the need of such information for the interpretation of the nervous control of ovulation and sexual behaviour we have investigated changes in tyrosine, dopamine, noradrenaline, tryptophan and 5-hydroxytryptamine concentrations during the oestrous cycle in the mouse.

The stage of the oestrous cycle in virgin albino mice (20–25 g) was established by vaginal smear immediately before the animals were killed by stunning and decapitation. The brains were removed and dissected on an ice-cold tile into the fore-brain (cortex), the mid-brain (thalamus, hypothalamus and striatum) and the hind-brain (corpora quadrigemina, pons, medulla and cerebellum). The dissection procedure gave highly reproducible results, with very low standard deviation when the weights of samples from 54 animals were compared. Noradrenaline and dopamine were determined by the method of Welch & Welch (1969), 5-hydroxytryptamine by the method of Snyder, Axelrod & Zweig (1965); tyrosine by the method of Waalkes & Udenfriend (1957) and tryptophan by the method of Hess & Udenfriend (1959).

Brain portions from three mice were pooled for the determinations. In the Tables, figures represent the mean ± s.e. nmol/g brain for six groups of mice. Statistical significance was calculated using the Student's *t*-test. The results show that changes in the concentrations of noradrenaline, dopamine and 5-HT, and their precursor amino-acids occur in both the fore- and mid-brain portions of the mouse brain during the oestrous cycle. These changes appear to be related to changes in sex hormone concentrations in the blood.

At dioestrus, the concentrations of 5-HT, noradrenaline and dopamine in the fore- and mid-brain are at their maxima. There is little hormonal activity at this time,

Table 1. *Tyrosine, dopamine and noradrenaline concentrations in the brains of mice at different stages of the oestrous cycle.*

	Tyrosine			Dopamine			Noradrenaline		
Brain portion	Fore	Mid	Hind	Fore	Mid	Hind	Fore	Mid	Hind
Dioestrus	72 ± 3	193 ± 10	104 ± 10	2·5 ± 0·2	8·2 ± 0·5	2·7 ± 0·5	2·3 ± 0·2	5·9 ± 0·2	2·5 ± 0·5
Proestrus	62 ± 5	*154 ± 9	1·5 ± 10	2·9 ± 0·4	*5·5 ± 0·6	2·2 ± 0·4	2·0 ± 0·3	4·9 ± 0·6	2·3 ± 0·3
Oestrus	58 ± 6	*141 ± 5	104 ± 11	*1·7 ± 0·2	**3·3 ± 0·4	2·6 ± 0·3	*1·0 ± 0·1	**2·7 ± 0·4	2·0 ± 0·3
Metoestrus	60 ± 7	166 ± 16	104 ± 10	2·3 ± 0·4	7·5 ± 0·4	2·4 ± 0·2	1·7 ± 0·3	*4·0 ± 0·6	2·1 ± 0·3

All figures are the mean ± s.e. of determinations on six groups in which the brains from three mice were pooled, expressed in nmol/g brain tissue. Statistical significance is shown as *P = 0·05, **P = 0·01.

Table 2. *Tryptophan and 5-hydroxytryptamine concentrations in the brains of mice at different stages of the oestrous cycle.*

	Tryptophan			5-hydroxytryptamine		
Brain portion	Fore	Mid	Hind	Fore	Mid	Hind
Dioestrus	31 ± 2·5	26 ± 1·5	21 ± 2·3	2·2 ± 0·4	8·7 ± 1·0	4·1 ± 0·6
Proestrus	26 ± 1·6	*16 ± 0·8	18 ± 1·3	2·0 ± 0·4	7·5 ± 0·8	3·7 ± 0·6
Oestrus	*19 ± 1·3	*14 ± 0·7	*15 ± 1·0	1·6 ± 0·2	*5·6 ± 0·8	3·9 ± 0·7
Metoestrus	*24 ± 1·6	*18 ± 1·2	20 ± 1·2	2·3 ± 0·3	7·2 ± 0·9	3·9 ± 0·6

All figures are the mean ± s.e. of determinations on six groups in which the brains from three mice were pooled, expressed in nmol/g brain tissue. Statistical significance is shown as *P = 0·05, **P = 0·01.

oestrogen and progesterone concentrations being low (Schwartz, 1969). During proestrus, the ovarian follicles mature under the influence of follicle-stimulating hormone and blood oestrogen concentrations rise steeply (Schwartz, 1969); mid-brain tyrosine, tryptophan and dopamine concentrations decrease significantly at this time. At oestrus, when ovulation is stimulated by luteinizing hormone, the concentrations of all three amines and their precursors are at their minima. During metoestrus, the corpora lutea in the ovary produce progesterone; amine and amino-acid concentrations begin to return to their dioestrus concentrations.

It has been shown that drugs which affect monoamine concentrations in the brain can produce ovulation in animals (Labhsetwar, 1971). The fact that changes in monoamine concentrations during oestrus are not specifically localized in the mid-brain (containing the hypothalamus), but also occur in the fore-brain, suggests that changes in monoamine concentrations may be important not only in the direct regulation of ovarian function but also in the initiation of oestrous behaviour. This suggestion is supported by the observations of Meyerson (1964, 1966, 1970) that oestrous behaviour can be elicited in ovariectomized animals by combinations of oestrogen with drugs affecting monoamine metabolism, and by the fact that cortical and limbic lesions abolish heat behaviour without affecting the pituitary-ovarian cycle (Beach, 1944). There is also some evidence that oestrogen and progesterone have direct effects on brain monoamine concentrations (Tonge & Greengrass, 1971).

It may be suggested that the well-established concept of a feed-back link between the hypothalamus and the ovary, controlling ovulation, should be extended to include a more wide-spread effect of ovarian hormones in the central nervous system, affecting behaviour. The growing acceptance of the monoamine theory of affective disorders (Coppen, 1967) invites speculation on the role of the sex hormone induced changes in brain monoamine concentrations in the aetiology of pre-menstrual, post-partum and menopausal depressions in women, since in all these conditions oestrogen and progesterone levels fluctuate widely.

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Isoprenaline stimulates α-adrenoceptors in bovine pulmonary blood vessels

Some interest has centred recently on the cardio-respiratory pharmacology of ruminants (Eyre 1969, 1970; Alexander, Eyre & others 1967, 1970: Aitken & Sanford 1969, 1970). It seems certain that the lung is an important anaphylactic shock organ of cattle, and in addition to the liberation of histamine (Eyre, 1971a) and 5-HT (Eyre: unpublished observations), dopamine is liberated from mast cells which may also participate in the pulmonary anaphylactic responses of this species (Eyre, 1971b). It was thus necessary to study adrenergic mechanisms in the bovine pulmonary musculature as part of a wider investigation.

Pulmonary artery, vein and bronchus were removed from the lungs of cattle 20 to 60 min after slaughter. Blood vessels were cut spirally and bronchi cut into rings before mounting in the usual way at 35° in a bath of 20 ml Krebs-Henseleit (1932) solution, gassed with 5% carbon dioxide in oxygen.

The blood vessel strips contracted to (ng/ml as base) histamine 50, 5-HT 2, acetylcholine 20, noradrenaline 100, phenylephrine and dopamine 500. Isoprenaline (5–50 ng/ml) relaxed the muscle preparations which were partially contracted in the presence of histamine, 5-HT or acetylcholine and this relaxant effect of isoprenaline was inhibited by the β-adrenoceptor blocking agent propranolol (100–200 ng/ml). Concentrations of isoprenaline $>1{\cdot}0$ μg/ml contracted the pulmonary artery and vein strips in the presence of propranolol 100 ng/ml. This contractile response of isoprenaline was abolished by the α-adrenoceptor blocking agent phentolamine (200–500 ng/ml).

The bronchial muscle contracted to histamine, 5-HT and acetylcholine whereas isoprenaline and dopamine caused relaxation. Noradrenaline and phenylephrine failed to elicit any response in bronchial muscle. The relaxant action of dopamine and isoprenaline was inhibited by propranolol (200 ng/ml). However, increasing concentrations of dopamine or isoprenaline, up to 50 μg/ml failed to contract the bronchial musculature.

It would appear that bovine pulmonary artery and vein possess both α- and β-adrenoceptors and that in the presence of propanolol, isoprenaline has an affinity for α-receptors which is comparable with that of noradrenaline or phenylephrine. Flacke, Osgood & Bendixen (1970) described a weak α action of isoprenaline on peripheral resistance in the dog. It may be that α effects of so called β-adrenergic agents have not been fully recognized.

Isoprenaline showed no tendency to constrict bovine bronchial muscle. Indeed neither noradrenaline nor phenylephrine had any action on the bronchial strip and it may be that the bovine airway musculature is devoid of α-adrenoceptors in contrast to such species as guinea-pig, rat, rabbit, cat (Fleisch, Maling & Brodie, 1970).

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A study of aqueous systems of purified non-ionic surfactant by membrane osmometry

The application of the membrane osmometry to the determination of number average micellar molecular weights ($\bar{M}_n$) has been reported by Coll (1969, 1970) and Attwood, Elworthy & Kayne (1969, 1970). We report the value of $\bar{M}_n$ of a purified sample of a non-ionic surfactant of pharmaceutical interest, polysorbate 80 and show that the effect of solubilizing nitrofurazone in micelles can be studied by membrane osmometry. Further, osmotic pressure measurements on the purified sample are compared with the commercial sample as well as a sample containing a known amount of polyoxyethylene glycol 600 (PEG 600).

A commercial sample of polysorbate 80 (polyoxyethylene-20-sorbitan mono-oleate) B.P.C. was purified by partitioning between 5N sodium chloride solution and ethyl acetate (Weibull, 1960). Samples were dried under passage of indifferent gas for 2 h at 30°/15 mm Hg followed by 1 h at 30°/3 mm Hg. Precipitated salts were filtered off through a sintered glass filter (G 3). Removal of contaminating substances was followed by thin-layer chromatography (Thakkar, Kuhn & Hall, 1967; Cerdas, Carlier & others, 1968). The extraction procedure removed polyoxyethylene glycols or polyoxyethylated sorbitans (or both). The saponification values for the purified and non-purified polysorbate 80 were 61·1 and 48·8 respectively; the hydroxyl values were 54·6 and 74·0 respectively. The critical micelle concentration (cmc) for the purified polysorbate 80 at 25° was found by the method of Becher (1962) to be 0·11 g/litre (extrapolated value). Nitrofurazone B.P.C. was found to be solubilized to an extent of $2{\cdot}32 \times 10^{-5}$ mol/g surfactant by purified polysorbate 80 aqueous solutions at 25·0° (±0·05°).

A Melabs CSM-2 recording membrane osmometer was used in these measurements at 25·0° (±0·1°). A cellulose membrane (prepared by drying a film from a 25% solution of cellulose acetate in acetone + dimethylformamide and treating the film for about 5 min in 70° water) was used for all the measurements. The molecular weight of a protein, cytochrome-C (mol. wt. 13 400), was found from the osmometer to be 15 000, thus showing that the membrane was non-permeable to molecules larger than 15 000. The accuracy of osmometer was ±0·1 cm of solvent.

In all experiments, solutions of concentration much larger than the cmc were placed on the solution side, and the solvent side was filled with a solution of 1 g/litre (several times the cmc). The number of monomers in equilibrium with micelles in the above solutions does not significantly increase with concentration (i.e. over the cmc). Hence the osmotic pressure arising from the monomers will be expected to be negligible. Since the membrane was found to be non-permeable to molecules of weight over 15 000, we did not consider any possibility of micelle diffusion ($\bar{M}_n \gg$

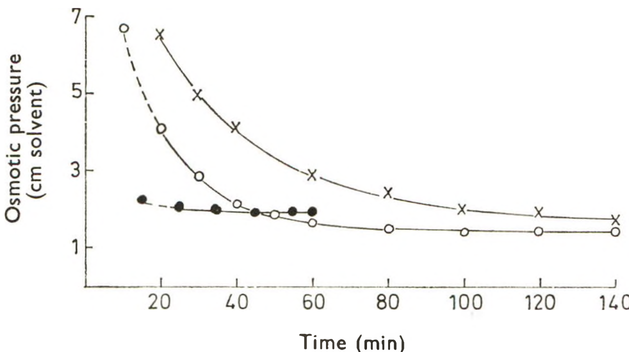

FIG. 1. Variation of osmotic pressure with time at 25° (concentration 9·00 g/litre). ● = purified polysorbate 80. ○ = mixture of purified polysorbate 80 and PEG 600 (4:1 w/w). × = non-purified polysorbate 80.

15 000). In the experiments with nitrofurazone, both the solutions in the solvent and solute side were saturated with nitrofurazone.

The experimentally measured osmotic pressure, P, between a sample solution of concentration c g/litre and a solvent of concentration c′ g/litre is given by (Coll, 1970; Attwood & others, 1970)

$$P = RT\,(c - c')/\bar{M}_n + RTB\,[(c - c')^2 + 2(c - c')\,(c' - cmc)]$$

where B is the second virial coefficient, RT has the usual values. The plots of $P/(c - c')$ versus $(c - c')$ were extrapolated to $c{=}c'$, and $\bar{M}_n$ was calculated, as given below:

$$[P/(c - c')]_{c=c'} = RT/\bar{M}_n + 2RTB(c' - cmc)$$

B had a value of $0{\cdot}5 \times 10^{-4}$ ml mol g^{-2}, and the correction term $2RTB(c' - cmc)$ gave a small correction of about 1% on $\bar{M}_n$.

The values of $\bar{M}_n$ for purified polysorbate 80 with and without nitrofurazone are 123 and 121 $\times 10^3$ respectively (at 25°). Thus the $\bar{M}_n$ does not change due to solubilization of nitrofurazone, within the experimental error (maximum 5%). This is to be expected, since only 2–3 molecules of nitrofurazone are solubilized per micelle.

The following systems were studied in order to determine the effect of impurities on the osmotic pressure: (*a*) purified polysorbate 80; (*b*) mixture of polysorbate 80 and PEG 600 (4:1 w/w); (*c*) non-purified polysorbate 80. The osmotic pressure curves against time are given in Fig. 1.

In the osmometer used, the rapid fall due to instrumental factors occurs within the first 5–10 min. Thereafter, any slow decrease is attributable to the diffusion of the solute. It can thus be concluded from Fig. 1, that the impurities in the sample are not completely solubilized in the micelles, thus giving rise to the initial decrease in osmotic pressure, when comparing the curves for purified and non-purified samples. Further, the curve for polysorbate 80 + PEG 600 mixture shows that PEG 600 gives rise to osmotic pressure and is not completely solubilized by the micelles, as expected from its hydrophilic nature.

These studies indicate that membrane osmometry of micellar systems can be useful in the determination of the effect of solubilization on the micellar molecular weight. Further it is possible to determine whether impurities are completely solubilized by the micelles.

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Further evidence on the question of polymorphism in aspirin

Several reports have recently appeared describing the preparation and properties of polymorphic forms of aspirin (Tawashi, 1968; Summers, Carless & Enever, 1970). Other reports have however suggested that the evidence for polymorphism so far presented is inconclusive and that the differences which exist between the various forms could be due to differences in crystal size and habit (Pfeiffer, 1971) or to crystal defects (Mitchell, Milaire & others, 1971). We wish to report some observations we have made during an investigation into the biopharmacy of salicylates which may help in resolving the conflict between these various viewpoints.

Preliminary studies using the procedures of Tawashi (1968) and Summers & others (1970) suggested that some salicylic acid was formed on dissolving aspirin in non-aqueous solvents. These observations were further investigated by equilibrating excess dry aspirin at 20° in sodium dried n-hexane and n-octane. Samples of solution were removed at various times and their absorbance measured over the range 270–320 nm using an SP 500 spectrophotometer. After 400 h 40 μg/ml of aspirin and 20 μg/ml of salicylic acid were found in the n-hexane solution and 30 μg/ml of aspirin and 10·5 μg/ml of salicylic acid in the n-octane using the Extinction Ratio method.

In view of the low solubility of aspirin in n-hexane at 20° it was necessary, in order to obtain an adequate yield, to reflux excess dry aspirin with n-hexane at 68° for 1½–2 h. After filtration, analysis of the hot solution showed the presence of approximately 600 μg/ml of aspirin and 60 μg/ml of salicylic acid. Portions of the hot solutions were allowed to stand at 0° or 20° for 14 h and the resulting crystals collected by filtration. The filtrates were then allowed to evaporate at the same temperatures to approximately a quarter of their original volume. After 200–350 h further samples of crystals were collected by filtration. The properties of the crystals obtained from these experiments are shown in Table 1 together with those of samples prepared by recrystallization from 96% ethanol and by sublimation at 118° onto a cold surface at 17° under various pressures.

The amount of salicylic acid present in the samples was determined both by the Extinction Ratio method in absolute ethanol and by a modification of the B.P.

limit test for salicylic acid in aspirin. The colour produced was measured at 530 nm and the amount of salicylic acid calculated from a calibration curve.

Thermal analyses of the samples were made using a Dupont 900 thermal analyser on 2–3 mg samples heated at 8°/min in air. Differential Thermal Analysis (DTA) peak temperatures are given in Table 1. Infrared spectroscopy showed no differences between the spectra of samples prepared from 96% ethanol and n-hexane.

Mitchell & Saville (1967) showed that samples of commercial aspirin have different intrinsic dissolution rates. The existence of polymorphic forms of aspirin, however, was first reported by Tawashi (1968), the stable form I (m.p. 143°) being obtained by crystallization from 95% ethanol and the less stable form II (m.p. 124°) by slow crystallization from n-hexane at room temperature. Form II dissolved more rapidly than form I and produced higher plasma levels after administration in man (Tawashi, 1969). Differences in the two forms were claimed to persist in dimethylformamide solution (Kildsig, Denbo & Peck, 1971). Summers & others (1970) described six polymorphs of aspirin obtained by sublimation or recrystallization under differing conditions from various organic solvents. These forms had different densities and melting points and underwent phase transformations in solution. Only minor differences were observed in their x-ray diffraction patterns however and Pfeiffer (1971) questioned the conclusion that aspirin polymorphism had been demonstrated. He suggested that the experimental observations could be explained by differences in crystal size and habit or to imperfections and stresses within the crystal. Mitchell & others (1971) agreed that the evidence for polymorphism was inconclusive and repeated their view that the differences could be due to crystal defects.

Table 1 indicates that all samples of aspirin prepared either by recrystallization or by sublimation are likely to contain traces of salicylic acid. The effect of impurities on the physicochemical properties of crystalline materials can vary depending on the

Table 1. *The melting-point and purity of aspirin samples prepared by various methods.*

Method of preparation	Salicylic acid (%) Extinction Ratio method	Salicylic acid (%) Modified B.P. Colour test	Melting point (°C) DTA	Melting point (°C) DSC*
Recrystallization from 96% ethanol at 20°	0·10	0·05	141	135
Recrystallization from a saturated solution at 68° in n-hexane				
After standing for 14 h at 20°†	0·4–1·4	0·4–1·0	135·3–138·6	—
After standing for 14 h at 0°†	0·5–2·4	0·5–2·3	134·0–138·6	—
Slow recrystallization from n-hexane at 20°†	0·6–1·0	0·8–1·0	126–132	123 & 114
Slow recrystallization from n-hexane at 0°†	0·4–2·0	0·4–2·1	128–136	129
Aspirin sublimed at the following pressures (mmHg): 12	60·0	65·0	115	For sublimed aspirin under vacuum mp = 108
1·6	23·9	24·3	115 & 127‡	
0·8	17·7	16·9	114 & 130‡	
0·05	1·3	2·0	136·6	

* The values quoted here were obtained by Summers & others (1970) using a Perkin Elmer Differential Scanning Calorimeter.

† The recrystallization experiments from n-hexane were repeated four times and the ranges quoted are for the two extreme results.

‡ Where two melting points are quoted, two peaks were observed on the DTA analysis.

mechanism of incorporation. An impurity may be captured by the crystal lattice forming solid solutions of various types. In such cases as little as 0·14% may be sufficient to distort the lattice (Khamskii, 1969). Alternatively it may be adsorbed onto internal or external surfaces or be present in the form of separate crystals. Another frequent phenomenon is the modification of crystal habit by impurities (Mullin, 1961). In all these processes the conditions of crystallization play an important role. Varying the conditions in our experiments produced samples with different melting points and different amounts of salicylic acid. Although individual samples were found to melt at a definite temperature, repeating the experiments under the same conditions yielded crystals melting at similar but not identical temperatures. These observations suggest that variations in the amount, location and bonding of the salicylic acid within the crystal can influence the melting point. Furthermore sublimed aspirin containing 16–25% of salicylic acid gave on thermal analysis two distinct peaks suggesting the existence of two or more types of association.

Since all the previously published phenomena attributed to polymorphism could be explained if salicylic acid had been present in the samples, and as significant differences in x-ray diffraction have not been found, it seems more reasonable to interpret the data as being due to the presence of this impurity rather than polymorphism. Further, unless salicylic acid can be excluded from aspirin samples polymorphism will be difficult to prove.

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