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Central hypotensive effect of L-3,4-dihydroxyphenylalanine in the rat

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Mean arterial blood pressure was recorded through in-dwelling arterial catheters in conscious normotensive Sprague-Dawley rats. L-3,4-Dihydroxyphenylalanine (L-dopa) was given in various doses intraperitoneally, alone and after pretreatment with an inhibitor of dopa decarboxylase, α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl) propionic acid (MK 485) or seryl-2,3,4-trihydroxybenzylhydrazine (Ro 4–4602). L-Dopa (50 mg/kg) produced a hypertensive response which was abolished by MK 485 (100 mg/kg). A larger dose of L-dopa (200 mg/kg) after MK 485 caused a significant lowering of blood pressure after 15–20 min. After Ro 4–4602 (400 + 200 mg/kg), injection of L-dopa (200 mg/kg) had no significant effect on blood pressure. The hypotensive response to L-dopa (200 mg/kg) after MK 485 was not influenced by the central dopamine receptor blocking agent, spiroperidol (0·1 mg/kg), but could be completely inhibited by the dopamine β-hydroxylase inhibitor, bis-(4-methyl-1-homopiperazinyl-thiocarbonyl)disulphide (FLA 63) (40 mg/kg). Pretreatment with protriptyline (10 mg/kg) completely blocked the hypotensive effect of L-dopa after MK 485. In correlative biochemical experiments, levels of noradrenaline and dopamine were determined in brain, heart and femoral muscle. L-Dopa (200 mg/kg) alone caused a significant increase of dopamine levels in all tissues. After MK 485 and Ro 4–4602 L-dopa did not significantly increase the levels of dopamine in heart or femoral muscle; however, brain dopamine levels were increased more than after L-dopa alone, but brain dopamine levels after Ro 4–4602 were significantly lower than after MK 485, indicating some central decarboxylase inhibition by Ro 4–4602. L-Dopa alone reduced the noradrenaline content of the heart and this effect was prevented by MK 485 and Ro 4–4602. The results show that decarboxylation of L-dopa in both the central and the peripheral nervous system leads to an increase in blood pressure. Decarboxylation of L-dopa in the central nervous system only results in a hypotensive response, provided that high amounts of dopamine are formed in the brain. This effect was prevented by an inhibitor of dopamine β-hydroxylase but not by a dopamine receptor blocker. Therefore, a central noradrenaline mechanism seems to be involved. The presence of an intact membrane pump in noradrenaline neurons may be essential since protriptyline also blocked the hypotensive action.

Administration of the catecholamine precursor L-3,4-dihydroxyphenylalanine (L-dopa) to experimental animals produces a syndrome which involves effects elicited both from the central and the peripheral nervous system (see e.g. Butcher & Engel, 1969a, b; Carlsson, 1969). Since L-dopa seems to be pharmacologically inert (Blaschko & Chruschiel, 1960; Carlsson, 1964), it may be assumed that its actions are mediated via its metabolites. Of particular interest are the catecholamines, dopamine and noradrenaline which may act centrally as well as peripherally.

Potent inhibitors of dopa decarboxylase in peripheral tissues, but with little effect

in the central nervous system, have previously been used to dissociate central and peripheral effects of the dopa analogue L-α-methyldopa (Henning 1968, 1969a) as well as L-dopa itself (Butcher & Engel, 1969a, b). We have examined the influence of L-dopa on blood pressure in conscious rats before and after pretreatment with α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl)propionic acid (MK 485), a decarboxylase inhibitor with minimimal central actions (Porter, Watson & others, 1962; Bartholini & Pletscher, 1969). The influence of another decarboxylase inhibitor, seryl-2,3,4-trihydroxybenzylhydrazine (Ro 4–4602) was also studied. This inhibitor, when given in sufficiently large doses, acts on both central and peripheral decarboxylase (see e.g. Henning, 1969a; Bartholini & Pletscher, 1969). Injection of L-dopa alone gave an increase in blood pressure which could be converted to a hypotensive response by pretreatment with MK 485; after pretreatment with Ro 4–4602 L-dopa had no effects on blood pressure. The hypotensive action was analysed further by studying the influence of an inhibitor of dopamine-β-hydroxylase, bis-(4-methyl-1-homopiperazinyl-thiocarbonyl)disulphide (FLA 63; Carlsson, Corrodi, Florvall, Ross & Sjöberg, unpublished data; cf. Svensson & Waldeck, 1969), a central dopamine receptor blocking agent, spiroperidol (Andén, Butcher, & others, 1970) and protriptyline. Part of the results have been presented in a preliminary report (Henning & Rubenson, 1970).

EXPERIMENTAL

The mean arterial blood pressure was recorded in conscious unrestrained male Sprague-Dawley rats, 250–350 g, through in-dwelling arterial catheters connected to Statham pressure transducers writing on a Grass Polygraph (Popovic & Popovic, 1960). For technical details see Henning (1969b). The blood pressure values represent averages of the recordings for the 10 min periods immediately before the administration of the drugs except the values after L-dopa which are averages of the pressure for 15–20 min after the injection.

In the biochemical studies, rats of corresponding body weight were used. Noradrenaline was determined by the method of Bertler, Carlsson & Rosengren (1958), dopamine as described by Carlsson & Lindqvist (1962). Each analysis was made on pooled organs from two animals.

The drugs used were L-3,4-dihydroxyphenylalanine (L-dopa), α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl)-propionic acid (MK 485), bis-(4-methyl-1-homopiperazinyl-thiocarbonyl) disulphide (FLA 63), spiroperidol, seryl-2,3,4-trihydroxybenzylhydrazine (Ro 4–4602). All drugs were administered intraperitonally. Solutions were prepared immediately before injection. MK 485 and Ro 4–4602 were dissolved in 0·9% saline. L-Dopa was dissolved in slightly warmed 0·9% saline (pH 5–6). The spiroperidol was dissolved in a few drops of acetic acid and then diluted in 5·5% glucose solution to a concentration of 0·02 mg/ml. FLA 63 was dissolved in 0·9% saline (pH 6·7). Doses and time intervals are given with the results. Tests of significance were by Student's *t*-test and analysis of variance with one or two independent criteria of classification. *P* values less than 0·05 were regarded as significant.

RESULTS

Blood pressure experiments

L-dopa. A significant ($P < 0{\cdot}005$) rise in mean arterial blood pressure was observed 20 min after injection of L-dopa, 50 mg/kg, the maximum being reached after about

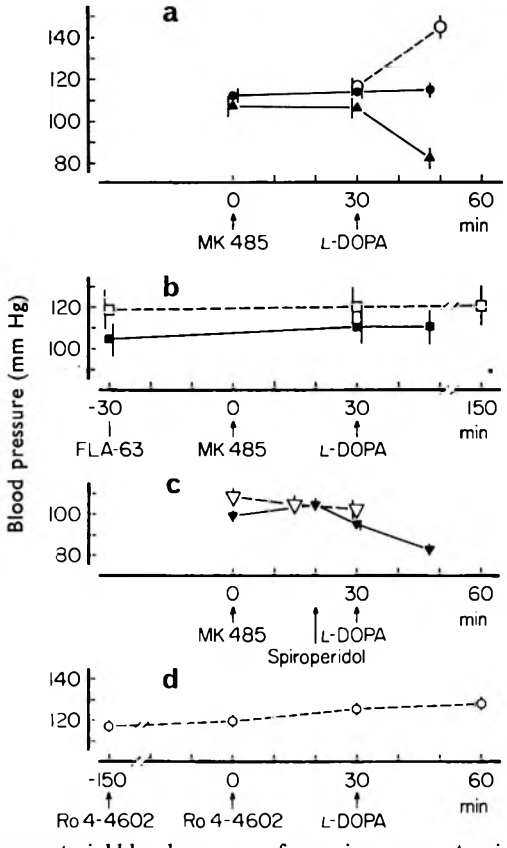

FIG. 1. Changes in mean arterial blood pressure of conscious normotensive rats after i.p. injection of various drugs. The blood pressure values represent averages of the recordings during the 10 min period immediately preceding the drug indicated except the values after L-dopa which are averages of the pressure for 15–20 min after the injection. (a) L-Dopa, 50 mg/kg ○ (9 exp.); L-dopa, 50 mg/kg, 30 min after MK 485, 100 mg/kg ● (5 exp.); L-Dopa, 200 mg/kg, 30 min after MK 485, 100 mg/kg ▲ (13 exp.). (b) L-Dopa, 200 mg/kg, 30 min after MK 485, 100 mg/kg and 60 min after FLA 63, 40 mg/kg ■ (8 exp.); FLA 63, 40 mg/kg alone □ (4 exp.). (c) L-Dopa, 200 mg/kg, 10 min after spiroperidol, 0·1 mg/kg, and 30 min after MK 485, 100 mg/kg ▼ (4 exp.); spiroperidol, 0·1 mg/kg ▽ (5 exp.). (d) Two doses of Ro 4–4602, 400 and 200 mg/kg, 3 and 0·5 h respectively, before L-dopa, 200 mg/kg (4 exp.).

20 min (Fig. 1a). The duration of the increase in blood pressure was over 60 min in all experiments. The animals showed piloerection and exophthalmus 15–30 min after the 50 mg/kg injection.

L-Dopa was also tested in 10, 20, 100 and 200 mg/kg doses; the lower doses either did not affect blood pressure or produced small increases. The higher doses consistently produced hypertensive responses.

MK 485 + L-*dopa*. Injection of MK 485, 100 mg/kg did not influence the mean arterial blood pressure significantly in 30 min ($P > 0{\cdot}1$) (Fig. 1a). Thirty min after MK 485, injection of L-dopa (50 mg/kg) did not produce any significant change in blood pressure. When the dose of L-dopa was increased to 200 mg/kg after MK 485, a significant fall in blood pressure was observed in 15–20 min ($P<0{\cdot}001$). Shortly after injection of L-dopa the animals had slight piloerection and exophthalmus and were aggressive and had an increased spontaneous motility. These behavioural changes reached a maximum 30 min after the L-dopa.

Ro 4–4602 + L-*dopa.* Four rats were given two doses of Ro 4–4602, 400 and 200 mg/kg, at 3 and 0·5 h, respectively before L-dopa (200 mg/kg). Mean arterial blood pressure was measured before the two doses of Ro 4–4602, before and 15–20 min after L-dopa. The results are shown in Fig. 1d. There were no significant changes in blood pressures ($P > 0·1$). In these experiments, the animals showed no clear behavioural changes after the injection of L-dopa.

FLA 63 + *MK* 485 + L-*dopa.* FLA 63 (40 mg/kg) did not have any significant effect on the mean arterial blood pressure in the 1–3 h after the injection (4 experiments; Fig. 1b).

In another series of experiments FLA 63 (40 mg/kg) was given 30 min before MK 485 (100 mg/kg). When L-dopa (200 mg/kg) was injected 30 min later there was no significant change in blood pressure ($P > 0·1$; Fig. 1b) and there were no signs of aggressiveness, but there was increased spontaneous motility with a predominance of stereotyped movements.

MK 485 + *spiroperidol* + L-*dopa.* Injection of spiroperidol (0·1 mg/kg) had no effect on mean arterial blood pressure ($P > 0·10$, 5 experiments; Fig. 1c), but the animals were sedated. After a pretreatment with MK 485 (100 mg/kg, 30 min before) and spiroperidol (0·1 mg/kg, 10 min before), L-dopa (200 mg/kg) caused a significant drop in mean arterial blood pressure ($P < 0·025$); there was very slight aggressiveness but a clear increase in spontaneous motility.

MK 485 + *protriptyline* + L-*dopa.* Four rats were given MK 485 (100 mg/kg) and protriptyline (10 mg/kg) 30 and 15 min, respectively, before L-dopa (200 mg/kg). The mean arterial blood pressure levels were as follows: before MK 485, 115 mm Hg (s.e. = 2·9); before protriptyline, 114 mm Hg (s.e. = 2·9); before L-dopa, 112 mm Hg (s.e. = 2·9); and 15–20 min after L-dopa, 116 mm Hg (s.e. = 2·9). The values are not significantly different from each other ($P > 0·1$).

Biochemical experiments

Tissue concentrations of dopamine and noradrenaline were determined after L-dopa alone and after pretreatment with MK 485 or Ro 4–4602. The animals were killed 1 h after the injection of L-dopa. The results are in Table 1.

L-Dopa alone (200 mg/kg) caused a pronounced increase in dopamine concentrations in heart and femoral muscle. There was also a marked increase in the levels of brain dopamine. The noradrenaline content of the heart, but not femoral muscle or brain, was significantly ($P < 0·001$) decreased.

Pretreatment with MK 485 30 min before L-dopa largely prevented the increase of dopamine in the heart and femoral muscle, but the increase in brain dopamine was more pronounced than after injection of L-dopa alone ($P < 0·001$). There were also slightly increased noradrenaline concentrations in the heart and femoral muscle ($P < 0·05$ and $P < 0·005$, respectively) but not in brain.

As with MK 485, Ro 4–4602 before L-dopa (doses and time intervals as in the blood pressure experiments) prevented the accumulation of dopamine significantly in peripheral tissues (heart, femoral muscle). The increase in brain dopamine was not significantly different from after L-dopa alone. However, the increase in brain dopamine after Ro 4–4602 was significantly ($P < 0·001$) less than after MK 485 plus L-dopa.

Table 1. *Levels of dopamine and noradrenaline in various tissues of normal rats and rats treated with* L-*dopa* (200 mg/kg i.p.) *or the same dose of* L-*dopa* 30 *min after MK* 485 (100 mg/kg, i.p.). The values are means with standard errors in μg/g and the number of analyses of 2 pooled organs are given in parentheses. *P* values were calculated by analysis of variance.

	Control	L-Dopa	MK 485+ L-dopa	Ro 4-4602 +L-dopa
Dopamine				
Brain	0·61 0·158 (5)	1·93 0·204 (3)	3·71 0·158 (5)	2·44 0·158 (3)
	< 0·001	< 0·001	< 0·001	
	< 0·001			
	> 0·10			
	< 0·001			
Hear	0·07 0·287 (5)	2·67 0·262 (6)	0·36 0·287 (5)	0·12 0·287 (5)
	< 0·001	< 0·001	> 0·10	
	> 0·10			
	< 0·001			
	> 0·10			
Femoral muscle	0·01 0·051 (5)	0·71 0·047 (6)	0·07 0·051 (5)	0·04 0·051 (5)
	< 0·001	< 0·001	> 0·10	
	> 0·10			
	< 0·001			
	> 0·10			
Noradrenaline				
Brain	0·38 0·029 (5)	0·38 0·038 (3)	0·40 0·029 (5)	0·38 0·029 (5)
	> 0·10	> 0·10	> 0·10	
	> 0·10			
	> 0·10			
	> 0·10			
Heart	0·96 0·047 (6)	0·53 0·047 (6)	1·13 0·057 (4)	0·83 0·051 (5)
	< 0·001	< 0·001	< 0·005	
	< 0·05			
	< 0·001			
	> 0·10			
Femoral muscle	0·10 0·006 (5)	0·09 0·005 (6)	0·12 0·006 (5)	0·07 0·006 (5)
	> 0·10	< 0·001	< 0·001	
	< 0·005			
	< 0·025			
	< 0·005			

Effects of dopamine on blood pressure

To examine the influence of dopamine on blood pressure, the amine was infused intravenously in graded doses in 4 conscious rats. At rates of 0·004 to 4 μg/min, there were no effects on blood pressure but at rates of 4 to 20 μg/min there were pressure responses.

DISCUSSION

Administration of L-dopa resulted in a pronounced increase in dopamine in sympathetically innervated tissues and a marked increase in brain concentrations. At the same time, L-dopa significantly reduced the noradrenaline content of the heart but not of the brain. Essentially similar findings have been reported previously (Bartholini, Burkard & others, 1967; Bartholini, da Prada & Pletscher, 1968; Bartholini & Pletscher, 1968; Butcher & Engel, 1969a, b). The blood pressure experiments revealed that L-dopa regularly elicited a hypertensive response in conscious rats. Previous studies have mostly been made in anaesthetized animals; the results are complex and indicate a wide species variation (Holtz & Credner, 1942; Dengler & Reichel, 1958; Gaillard, Schaeppi & Tissot, 1969; Pruss & McGill, 1969).

In animals pretreated with an inhibitor of peripheral decarboxylase (MK 485), the effect of L-dopa in increasing dopamine in peripheral tissues was almost completely prevented. Further, the effect of L-dopa in lowering heart noradrenaline was blocked, significantly indicating that this effect is due to a displacement of noradrenaline by dopamine formed from L-dopa. This mechanism is the same as that proposed for the noradrenaline-lowering effects of α-methyldopa and α-methyl-*m*-tyrosine which is also markedly reduced after decarboxylase inhibition (Carlsson & Lindqvist, 1962; Udenfriend & Zaltzman-Nirenberg, 1962; Gessa, Costa & others, 1962; Levine & Sjoerdsma, 1964; Henning, 1969a). In our experiments L-dopa increased brain dopamine levels significantly more after inhibition of peripheral decarboxylase, which confirms the results of other investigators (Bartholini & Pletscher, 1968; Butcher & Engel, 1969b) and is believed to result from an increased availability of L-dopa to the brain and, hence, an increased formation of dopamine.

The increase in blood pressure produced by L-dopa (50 mg/kg) was completely blocked by the peripheral decarboxylase inhibitor (MK 485). When the dose of L-dopa was increased, there was a significant fall in blood pressure. In contrast, the same dose of L-dopa had no effect on blood pressure after pretreatment with Ro 4–4602. Previous studies indicate that this compound is an effective inhibitor of both central and peripheral decarboxylase (see p. 554) although in our experiments Ro 4–4602 did not prevent the increase of brain dopamine produced by L-dopa. However, significantly more dopamine was formed in the brain after pretreatment with MK 485, indicating a significant degree of decarboxylase inhibition by Ro 4–4602 in the brain. Neither of the two decarboxylase inhibitors alone had any effect on blood pressure, as shown previously for corresponding time intervals (Henning, 1969a).

The interaction of the two decarboxylase inhibitors with the effects of L-dopa on blood pressure may be interpreted as follows. The hypertensive response seen after L-dopa alone is probably mediated through peripheral adrenergic mechanisms. Possibly, dopamine is involved, acting either directly by activation of the adrenergic receptors or indirectly by displacement of endogenous noradrenaline. The hypotensive response which is unmasked after inhibition of the peripheral decarboxylation of a

large dose of L-dopa is probably not due to peripheral actions of dopamine or noradrenaline though dopamine may have a dilator action in certain vascular beds in some species (see e.g. Holtz & Palm, 1966). The formation of dopamine in peripheral tissues was almost completely prevented by the decarboxylase inhibitor and in experiments with intravenous dopamine infusions in rats over a wide dose range, only hypertensive responses were encountered. Therefore, it seems most likely that an action of L-dopa metabolites in the central nervous system would explain the hypotensive effect of L-dopa after peripheral decarboxylase inhibition. Apparently, very high levels of such metabolites are required since a hypotensive response was associated only with the high dopamine levels produced after MK 485 pretreatment.

After pretreatment of the animal with an inhibitor of dopamine β-hydroxylase both the hypotensive response and the symptoms of aggressiveness produced by L-dopa were absent. In the dose used, FLA 63 markedly inhibited the β-hydroxylase (Svensson & Waldeck, 1969) and therefore the formation of noradrenaline from L-dopa was presumably prevented to a large extent. If this is so, an activation of noradrenergic mechanisms in the brain may account for the hypotension after L-dopa plus peripheral decarboxylase inhibition. A central noradrenaline receptor blocking action of FLA 63 is unlikely since this drug does not influence the noradrenaline receptors in the spinal cord in the rat (Andén, unpublished experiments). The lack of effect of spiroperidol, which in the dose used appears to block only central dopamine receptors in the rat (Andén, Butcher & others, 1970), could also be explained by a central noradrenergic activation by L-dopa metabolites. The antihypertensive drug clonidine (St 155) has recently been shown to have a central noradrenaline receptor stimulating action (Andén, Corrodi & others, 1970).

Pretreatment with protriptyline, which prevents the uptake of catecholamines in noradrenergic neurons (Carlsson, Fuxe & others, 1966; Jonason, 1969), blocked the hypotensive effect of L-dopa after peripheral decarboxylase inhibition. Assuming that the action of L-dopa in these experiments is mediated through central noradrenergic activation, the effect of protriptyline may be explained by a reduction in the neuronal uptake of dopamine. This could lead to a decreased availability of substrate for noradrenaline formation in these neurons, or to a diminished release of this amine, or both mechanisms in combination. It appears unlikely that the slight anticholinergic effect of protriptyline is of any relevance since unpublished experiments have shown that pretreatment with atropine sulphate (10 mg/kg) did not influence the hypotension after L-dopa plus MK 485. Another possible explanation is that protriptyline may exert a central noradrenaline receptor blocking effect.

The demonstration of a centrally mediated hypotensive response to L-dopa may have several important implications. As mentioned previously, L-dopa alone may cause lowering of blood pressure. During long-term oral L-dopa treatment of patients with Parkinson's disease, several investigators have noted episodes of hypotension, sometimes mainly of the postural type (Calne, Stern & others, 1969; Cotzias, Papavasiliou & Gellene, 1969; Yahr, Duvoisin & others, 1969). This may be related to an action of the drug of a similar nature to that found in rats pretreated with a decarboxylase inhibitor. Another indication is that similar effects could account for the effects of structural analogues of L-dopa. In preliminary experiments we have found that *m*-tyrosine behaves like L-dopa. However, this is not so with α-methyl-*m*-tyrosine which lacks hypotensive effects in the rat (Henning, 1967). α-Methyldopa,

which under certain conditions may produce hypertensive reactions, has previously been shown to retain its hypotensive properties after peripheral decarboxylase inhibition (Henning, 1969a). Further, preliminary experiments have shown that the hypotensive action of α-methyldopa is blocked after pretreatment with an inhibitor of dopamine β-hydroxylase. Taken together, these findings may indicate that L-dopa as well as its α-methylated derivative both act by activating central noradrenergic mechanisms.

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The effects of calcium on adrenergic neuron blockade

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The effects of increasing extracellular calcium were investigated on the responses to sympathetic nerve stimulation of three isolated organs; rabbit ileum, guinea-pig vas deferens and rabbit ear artery. A rise in the calcium concentration increased the responses of the ileum to low frequency stimulation, the maximum increase being obtained at 8·8 mM calcium. After partial blockade by guanethidine of the responses of the ileum to high frequency stimulation, raised calcium concentrations again increased the responses. The increase was similar in guanethidine-treated and untreated preparations and the maximum increase in both was obtained using 8·8 mM calcium. In the vas deferens and rabbit ear artery preparations an increase in extracellular calcium did not antagonize the blocking action of guanethidine. These experiments do not therefore support the theory that guanethidine acts by preventing the entry of calcium into the sympathetic nerve endings.

The effects of calcium on the release of noradrenaline by sympathetic nerve endings have been studied by several workers using a variety of isolated organ preparations. Kuriyama (1964) provided strong evidence that calcium was necessary for the release of noradrenaline by the sympathetic nerves to the guinea-pig vas deferens. Other evidence to support this postulated role of calcium has been obtained using rabbit ileum (Burn & Gibbons, 1964), cat spleen (Kirpekar & Misu, 1967), rabbit colon (Boullin, 1967) and rabbit ear arteries (Farmer & Campbell, 1967). Burn & Welsh (1967) found that after the responses of the rabbit ileum to sympathetic nerve stimulation had been partially blocked by guanethidine, they could be restored by raising the extracellular calcium concentration. These observations were extended by Kirpekar, Wakade & others (1969) who suggested that adrenergic neuron blockade by guanethidine was due to prevention of the access of calcium to its site of action in the sympathetic nerve ending.

The object of the present experiments was to examine the effects of raising the calcium concentration on adrenergic neuron blockade due to guanethidine in three isolated organs: rabbit ileum, rabbit ear artery and guinea-pig vas deferens.

EXPERIMENTAL

Rabbit ileum

Segments of rabbit ileum with their sympathetic nerves were prepared by the method of Finkleman (1930). A length of ileum 2–3 cm long was set up in a 25 ml bath containing McEwen (1956) solution at 35° and gassed with 5% carbon dioxide in oxygen. Movements of the ileum were recorded with an isotonic frontal writing

lever exerting a tension of 2 g. The periarterial nerves were stimulated with bipolar platinum electrodes for 15 s every 2 min (or for 30 s every 4 min) at frequencies of 3–40 pulses/s using 2 ms pulses at supramaximal voltage.

Guinea-pig vas deferens

The preparation of Huković (1961) was used. The vas deferens was set up in a 25 ml organ bath containing McEwen (1956) solution at 35° gassed with 5% carbon dioxide in oxygen. The hypogastric nerve was stimulated with bipolar platinum electrodes for 5 s every 2 min using 2 ms pulses at 2–20 pulses/s and supramaximal voltage. Contractions of the vas deferens were recorded using an isotonic frontal-writing lever producing four times magnification and exerting a tension of 0·5 g.

Rabbit ear artery

The preparation was dissected and set up as described by de la Lande & Rand (1965). The artery was perfused with McEwen (1956) solution at 37° and gassed with 5% carbon dioxide in oxygen. The flow of perfusion fluid was maintained with a constant volume roller pump at a rate of 6 ml/min and the perfusion pressure was measured using a pressure transducer and a Devices recorder. The periarterial sympathetic nerves were stimulated by means of bipolar platinum ring electrodes placed on the upper end of the vessel. Stimulation was at a rate of 10–20 pulses/s for 5 s every 2 min using 2 ms pulses and supramaximal voltage.

RESULTS

Rabbit ileum

Fig. 1 shows the blocking action of guanethidine on the responses of the rabbit ileum to sympathetic nerve stimulation and reversal of the blockade when the concentration of calcium in the bath was doubled.

In nine experiments, guanethidine (0·25 to 0·5 μg/ml), in contact with the preparation for 60 min, reduced the responses to stimulation of the sympathetic nerves at 20/s from complete inhibition of the pendular movements to 22·1% ± 1·9% (mean ± s.e.) inhibition. When the calcium content of the bath fluid was raised the responses of the preparation to sympathetic nerve stimulation were increased, a maximal

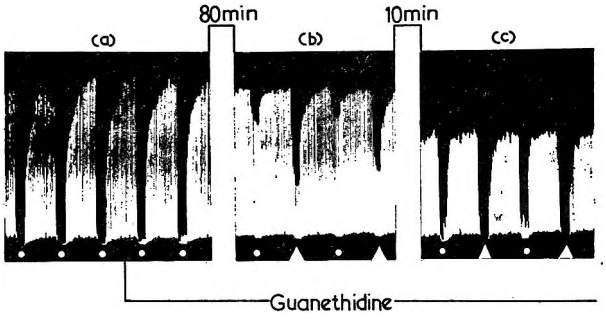

FIG. 1. Rabbit ileum. Effects of guanethidine on responses to stimulation of the sympathetic nerves for 30 s every 4 min using 2 ms pulses at frequencies of 20/s (●) and 40/s (△). In (*a*) and (b) McEwen solution containing 2·2 mM calcium was used. In (c) the calcium concentration was increased to 4·4 mM. Guanethidine dose: 0·4 μg/ml.

increase in the response being obtained in the presence of 8·8 mM calcium. The results of these experiments are shown in Fig. 2a.

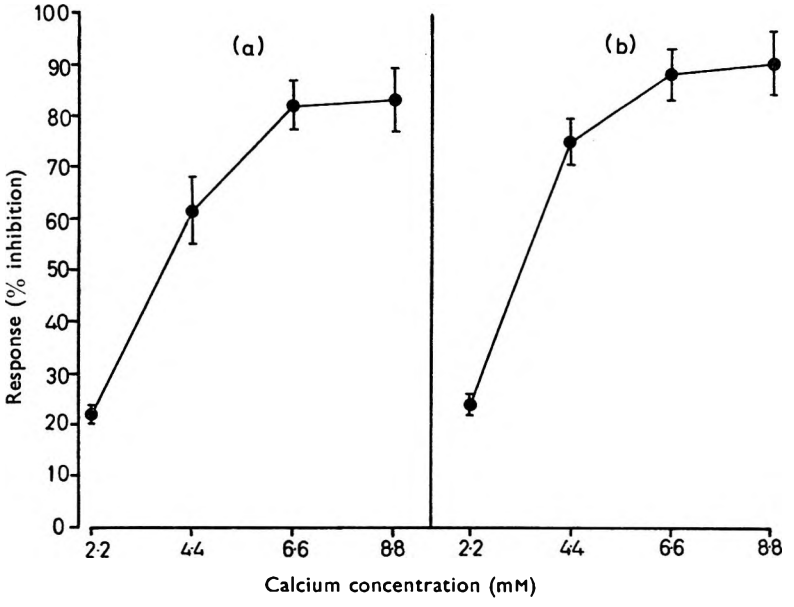

FIG. 2. Rabbit ileum. Responses (percentage inhibition of pendular movements) produced by stimulation of the sympathetic nerves using 2 ms pulses at frequencies stated and supramaximal voltage for 15 s every 2 min. (a) Stimulation at 20/s after exposure to guanethidine (0·25–0·5 μg/ml) for 60 min. (b) Stimulation at between 3/s and 6/s in the absence of guanethidine.

The responses to sympathetic nerve stimulation in the presence of various concentrations of calcium were also investigated in preparations which had not been treated with guanethidine. In twelve experiments the frequency of stimulation was adjusted to produce approximately 25% inhibition of the pendular movements. These responses were therefore similar in size to those obtained previously at a frequency of 20/s after exposure to guanethidine. When the calcium concentration was raised the responses were increased by an amount which was of the same order as in the guanethidine-treated preparations (Fig. 2b). The maximum responses in these preparations also were obtained at a calcium concentration of 8·8 mM. Four experiments were made in which guanethidine was added in the presence of 8·8 mM calcium. The concentration of guanethidine required to block the responses to sympathetic nerve stimulation in these experiments was between 0·25 and 0·5 μg/ml, i.e. the same as the concentration which blocked responses in the presence of 2·2 mM calcium. The blockade produced by guanethidine in the presence of 8·8 mM calcium could not be reversed by raising the calcium concentration still further. As shown in Fig. 3 calcium concentrations of up to 22 mM had no effect on the blocking action of guanethidine. Calcium concentrations higher than 22 mM were not used because of precipitation of calcium above this level.

In four experiments an increase in the calcium concentration from 2·2 to 4·4 mM also produced a small increase in the response of the ileum to added noradrenaline. However, this was insignificant compared with the increase in the response to

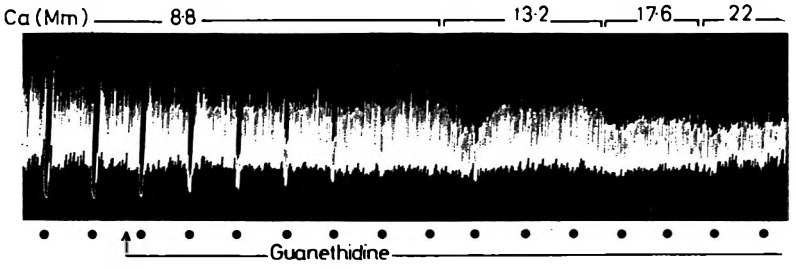

FIG. 3. Rabbit ileum. Effect of guanethidine (0·5 μg/ml) on responses of the rabbit ileum to stimulation with 2 ms pulses at a rate of 4/s for 15 s every 2 min.

sympathetic nerve stimulation. This confirmed the finding of Burn & Gibbons (1964) that the enhancement of the responses to sympathetic nerve stimulation could not be explained by supersensitivity to noradrenaline.

Guinea-pig vas deferens

Responses of the vas deferens to stimulation of the hypogastric nerve were recorded in the presence of calcium concentrations from 1·1 to 13·2 mM. In five preparations out of six the optimal calcium concentration was found to be 2·2 mM (Fig. 4) and in

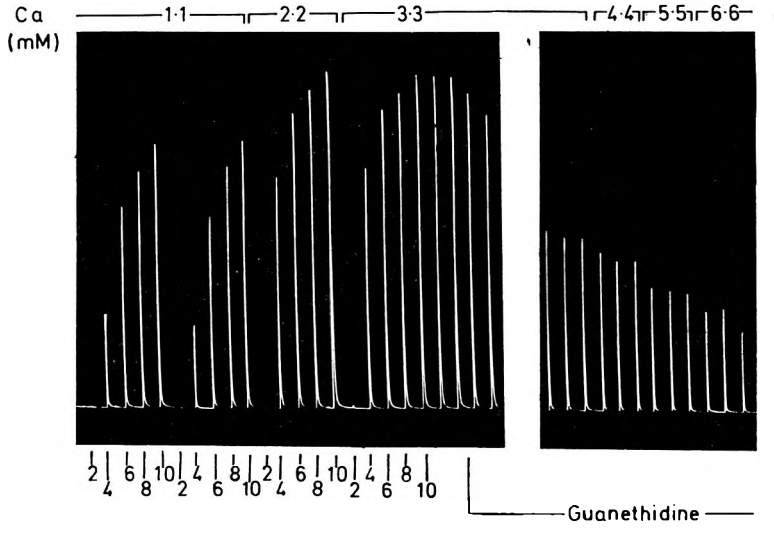

FIG. 4. Guinea-pig vas deferens. Responses to stimulation of the hypogastric nerve at rates of 2–10/s (responses where no frequency is indicated are to 10/s) for 5 s every 2 min using 2 ms pulses at maximal voltage. The record shows the effect of guanethidine (1 μg/ml) at various calcium concentrations. The right and left hand records are separated by an interval of 30 min.

one experiment it was 4·4 mM. In the experiment shown in Fig. 4 guanethidine (1 μg/ml) was added in the presence of a supramaximal concentration of calcium (3·3 mM). The responses were reduced by 50% in about 30 min, and the calcium concentration was then raised by 1·1 mM steps to 6·6 mM. This increase in calcium concentration did not diminish the blocking action of guanethidine. Similar results were obtained in four other experiments carried out in this way. When the calcium

concentration was increased above 6·6 mM, the blocking action of guanethidine appeared to be intensified (Fig. 5). However, the responses to hypogastric nerve stimulation were also reduced by calcium concentrations of 8·8 to 13·2 mM in preparations not treated with guanethidine.

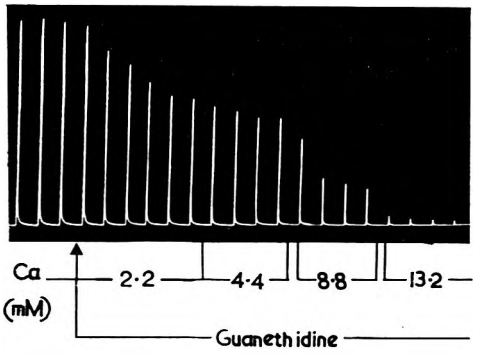

FIG. 5. Guinea-pig vas deferens. Responses to stimulation of the hypogastric nerve for 5 s every 2 min using 2 ms pulses at a frequency of 16/s. The record shows the effect of varying the calcium concentration on the action of guanethidine (0·5 μg/ml).

Rabbit ear artery

In experiments using the rabbit isolated ear artery a partial blockade of the responses to sympathetic nerve stimulation was produced by perfusing with 0·5–1 μg/ml guanethidine. An increase in the calcium concentration of the perfusion fluid to 4·4 or 8·8 mM had no effect on the blockade produced by guanethidine. A further increase in calcium concentration to 13·2 mM reduced the responses (Fig. 6).

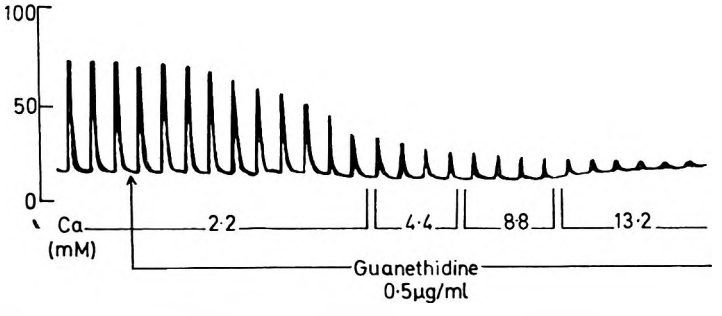

FIG. 6. Rabbit ear artery. Responses to stimulation of the periarterial sympathetic nerves for 5 s every 2 min using 2 ms pulses at a frequency of 14/s. The scale indicates the perfusion pressure in mm Hg. The record shows the effect of increasing the calcium concentration on the adrenergic neuron blockade produced by guanethidine (0·5 μg/ml).

DISCUSSION

The experiments reported here, using the rabbit ileum, confirm the findings of Burn & Welsh (1967). Thus, after a partial blockade of the responses of the ileum to sympathetic nerve stimulation by guanethidine, the responses could be restored to their original size by a 2- to 4-fold increase in the concentration of calcium in the organ bath. This led Burn & Welsh (1967) to suggest that guanethidine acted by preventing the entry of calcium into the sympathetic nerve ending, a process which is believed to be necessary for the release of noradrenaline from sympathetic nerves

(Kirpekar & Misu, 1967; Boullin, 1967). However, doubling the calcium concentration of the bath fluid (from 2·2 to 4·4 mM) increases responses of the rabbit ileum to sympathetic nerve stimulation even in the absence of guanethidine by providing an optimal calcium concentration. It therefore seems possible that the apparent reversal of the blocking action of guanethidine is due simply to physiological antagonism. The enhancement of sympathetic inhibition by providing optimal calcium concentration merely opposes the effect of guanethidine.

In the present experiments an approximately 25% inhibition of the ileum was obtained either by low frequency stimulation of the sympathetic nerves in the absence of guanethidine or by high frequency stimulation after 60 min exposure to guanethidine. The amounts by which these responses were increased when the calcium concentration was raised from 2·2 to 4·4, 6·6 and 8·8 mM were similar whether or not guanethidine was present. In some experiments, pieces of ileum were set up in McEwen solution containing 8·8 mM calcium, the optimal concentration for responses to sympathetic nerve stimulation in this preparation. In spite of the high calcium content of the bath fluid, guanethidine blocked the responses in the same concentration (0·25 to 0·5 μg/ml) as in 2·2 mM calcium, and a further increase in calcium concentration to 22 mM produced no reversal of the blockade.

Experiments using the isolated hypogastric nerve—vas deferens of the guinea-pig also provided no evidence for a specific antagonism between calcium and guanethidine. This preparation differed from the rabbit ileum in having a lower calcium requirement for the production of maximal responses to sympathetic nerve stimulation. In the vas deferens, maximal responses were obtained in McEwen solution containing the usual calcium concentration of 2·2 mM. When experiments were made using a supramaximal calcium concentration (3·3 mM) no reversal of the blocking action of guanethidine could be produced by further increases in calcium concentration. When the calcium concentration was raised to 8·8 mM or above, the contractions of the vas deferens were depressed whether or not guanethidine was present. This may be due to the stabilizing effect of calcium on the smooth muscle membrane. These experiments with the vas deferens are more difficult to interpret because the hypogastric nerve is preganglionic and the effect of calcium on ganglionic transmission must be taken into account.

A similar lack of antagonism between calcium and guanethidine was seen using the isolated artery preparation. This preparation has been shown by Farmer & Campbell (1967) to respond maximally to sympathetic nerve stimulation when perfused with a solution containing 4·4 mM calcium. Thus, if the antagonism between calcium and guanethidine is "physiological" (i.e. they are producing opposing effects) a two-fold increase in the calcium content of the McEwen solution might be expected to oppose the blocking action of guanethidine, whereas higher calcium concentrations would have no further effect. In the above experiments, however, increases in calcium concentration up to 13·2 mM did not antagonize the action of guanethidine.

The experiments reported here show that the optimal calcium concentration for responses of isolated organs to sympathetic nerve stimulation varies from one preparation to another. Thus the responses of the vas deferens to sympathetic stimulation were not enhanced by increased calcium, since McEwen solution in which the preparation is set up, already contains the optimal calcium concentration.

However, this solution contains only 25% of the optimal concentration for the responses of the rabbit ileum to sympathetic nerve stimulation, so that a rise in calcium concentration increases these responses. These observations suggest that the reversal of the blocking action of guanethidine by calcium is due to physiological antagonism and do not support the theory that guanethidine acts by limiting the access of calcium to its site of action in the sympathetic nerve endings.

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Reduction of gastric acid secretion and ulcer formation by 3,3-dimethyl-1-(3-methylaminopropyl)-1-phenylphthalan (Lu 3-010); an inhibitor of noradrenaline uptake

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Lu 3–010 [3,3-dimethyl-1-(3-methylaminopropyl)-1-phenylphthalan], administered intraperitoneally, blocks the uptake of [^{3}H]noradrenaline into the mouse and rat heart and has an activity 5 times greater than imipramine and comparable to that of desipramine. Lu 3–010 inhibits basal gastric acid secretion in the rat and is 4 and 2 times more potent than imipramine and desipramine, respectively. The drug is about 9 times more potent than desipramine in preventing the pentagastrin-induced stimulation of gastric acid secretion in the rat. Lu 3–010 reduces the incidence of stress-induced gastric lesions in the rat, exhibiting an ED50 $\pm$ s.e. of 6·3 $\pm$ 1·4 mg/kg, and at 10 mg/kg, ulcer development in the 17-hour Shay test is reduced by 50%.

Noradrenaline is mainly inactivated through its uptake into the storage sites of sympathetic nerve endings (Kopin, Hertting & Gordon, 1962; Chidsey, Kahler, Kelminson & Braunwald, 1963; Thoenen, Huerlimann & Haefely, 1964). Blockade of this uptake process, which is at the level of the cell membrane, could lead to a higher level of noradrenaline at the receptor sites and thus result in a potentiation of its effects on the effector organs. Various drugs which block this uptake process potentiate the pharmacological effects of noradrenaline (Haefely, Huerlimann & Thoenen, 1964). The drug 3,3-dimethyl-1-(3-methylaminopropyl)-1-phenylphthalan (I; Lu 3–010) blocks the uptake process and, since it is devoid of cholinergic activity, it appears to be the most specific inhibitor of this uptake mechanism (Waldeck, 1968). Various related pharmacological activities of Lu 3–010 are reported here.

EXPERIMENTAL

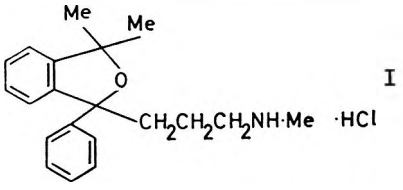
Radioactive noradrenaline levels

Male albino mice, 23–25 g, or rats, 60–80 g (Canadian Breeding Laboratories) were injected in the tail vein with 1·5 μCi ($\pm$)-7-[^{3}H]noradrenaline* HCl (Radiochemical Centre, Amersham) in 0·25 ml of a solution of 0·75% sodium chloride and 0·01N HCl. Drugs were injected intraperitoneally in 0·5 ml bidistilled water; the doses refer to the salt of the drug.

The tissue samples were homogenized in ice-cold 0·4N perchloric acid and centrifuged. A portion of the supernatant fluid was transferred to a vial containing a mixture of 1 ml methanol, 3 ml ethanol and 10 ml toluene phosphor [0·4% 2,5-diphenyloxazole and 0·005% 1,4-bis(5-phenyloxazol-2-yl)benzene] and the total radioactivity was measured by liquid scintillation counting (efficiency: 12%). The

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

radioactivity in the heart of the mouse (Burak & Draskoczy, 1964; Daly, Creveling & Witkop, 1966) and rat (Wurtman, Kopin & Axelrod, 1963) at times comparable to those of the present studies is almost entirely due to [^{3}H]noradrenaline.



I

Gastric acid secretion

The basal gastric acid secretory activity was determined by a modified method of Shay, Sun & Gruenstein (1954) in Charles River female albino rats (Canadian Breeding Laboratories; 170–190 g) caged individually and from which food had been withheld 48 h before pyloric ligation and drug administration. After the first 24 h of food deprivation the animals were given access to 8% sucrose in 0·2% sodium chloride for 8 h. Water was permitted freely except during the 8 h the animals were on sucrose and after drug treatment. The pylorus was ligated under ether anaesthesia and the sutured incision covered with collodion to prevent animals ingesting adhering blood. The stomachs were lavaged with 0·9% saline by No. 5 Argyle premature infant feeding tube until clear; immediately after, the drugs were given intraperitoneally. Three h after pyloric ligation, the animals were anaesthetized with ether, the stomachs tied at the oesophageal junction, removed, rinsed in water, opened along the greater curvature and the contents collected in centrifuge tubes. The amount of acid in the centrifuged gastric juice was determined by titration against 0·1N sodium hydroxide in a direct reading pH meter to pH 7·0. There were 4–9 animals in each group.

The effect on drug-stimulated secretory activity was determined according to Kim & Shore (1963) as modified by Levine (1965). Food was withheld from rats for 48 h but they received sucrose in the same way as did the animals used to determine basal gastric acid secretory activity. Under ether anaesthesia, the pylorus was ligated, the stomach lavaged with 0·9% saline until clear and the oesophagus ligated in the cervical region. Because of the oesophageal ligation, no collodion was necessary. Test drugs were given intraperitoneally immediately after the operations. Pentagastrin treatment (1 μg/kg, s.c.), according to Lippmann (1970), or saline, was started 20 min after the test drugs and continued at 20 min intervals for a total of 5 injections. There were 6–9 animals in each group. The animals were killed by a blow on the head 2 h after receiving the test drugs. The stomachs were removed in the same manner as described above and the gastric contents emptied into centrifuge tubes. The volumes of juice were noted and the stomachs were rinsed with bidistilled water to yield a final volume of 5 ml. The samples were centrifuged and titrated against 0·01N sodium hydroxide in a direct reading pH meter to pH 7·0 to obtain the total acid.

Development of gastric lesions

To assess the effects of the drugs on the development of multiple stress-induced gastric lesions, the method of Brodie & Valitski (1963) as modified by Senay & Levine (1967) was used. Food was withheld from male albino rats (150–180 g) for

24 h but water was freely available. Drugs were administered intraperitoneally to groups of 10 to 14 animals immediately before placing the rats into restrainers 45 min before exposure to a cold environment of 5°; the restrainer is made of plexiglass and restricts the movement of the rats to a minimum. In each experiment an equal number of untreated rats were run concurrently with treated rats. The cold exposure lasted 150 min and 1 h later the rats were killed with ether and their stomachs removed. The stomachs were opened along the larger curvature and then unfolded for inspection. Of the untreated rats, 80 to 100% developed gastric lesions consisting of one or several macroscopical erosions of the glandular mucosa often accompanied by haemorrhage. The results were scored on the basis of presence or absence of lesions regardless of their number and severity. The data were submitted to probit analysis and the ED50 (dose protecting 50% of the animals) was calculated.

Anti-ulcer activities were also assessed using the 17-h Shay test (Shay, Komarov & others, 1945). After the animals were fasted for 48 h the pylorus was ligated under ether anaesthesia and the abdominal cavity closed. Drugs were administered intraperitoneally immediately after the ligation. The rats were killed 17–19 h later and their stomachs tied at the cardia and removed. The stomachs were macroscopically examined and the severity of gastric lesion was graded according to the area showing glandular lesions. There were 5–10 animals in each group.

Student's *t*-test was used to evaluate all data except where otherwise noted.

RESULTS

The effects of the intraperitoneally-administered drugs on the uptake and release of [^{3}H]noradrenaline (^{3}H-NA) in the mouse and rat heart are shown in Table 1. In the mouse, the level of ^{3}H-NA, given after the drug, was decreased after Lu 3–010

Table 1. *Inhibition of uptake of* [^{3}*H*]*noradrenaline in the mouse and rat heart by Lu* 3–010

Drug	Dose mg/kg, i.p.	Radioactivity content: Drug given before ^{3}H-NA* counts/min g^{-1} ± s.e.	Inhibition %	Drug given after ^{3}H-NA† counts/min g^{-1} ± s.e.
Mouse				
Water		4631 ± 297		3443 ± 83
Lu 3–010	0·5	2284 ± 101 $P<0·001$	51	3451 ± 174
	0·25	2997 ± 202 $P<0·001$	35	
Imipramine ..	2·5	2861 ± 103 $P<0·001$	38	3643 ± 154
	1·0	3698 ± 162 $P<0·02$	20	
Desipramine ..	0·5	2687 ± 151 $P<0·001$	42	3747 ± 192
	0·25	3384 ± 112 $P<0·01$	27	
Rat				
Water		1630 ± 82		1501 ± 34
Lu 3–010	0·5	881 ± 29 $P<0·001$	46	1571 ± 49
	0·25	1263 ± 68 $P<0·01$	22	
Imipramine ..	2·5	733 ± 94 $P<0·001$	55	1677 ± 82
	1·0	1462 ± 111		
Desipramine ..	0·5	1068 ± 56 $P<0·001$	35	1523 ± 89
	0·25	1318 ± 103 $P<0·05$	19	

The animals were injected with drug 45 min before or after ^{3}H-NA and killed 75 min after the drug administration.

* There were 16 animals in the control and 8 in the treated groups.

† There were 10–12 animals in the control and 6–7 in the treated groups.

(51%), imipramine (38%) and desipramine (42%) at 0·5, 2·5 and 0·5 mg/kg, respectively. In the rat after the same doses of Lu 3–010, imipramine and desipramine there were declines of 46, 55 and 35%, respectively. Thus, in both animals Lu 3–010 was about 5 times more potent than imipramine and was similar in activity to desipramine.

In both the mouse and rat there were no reductions in heart ^{3}H-NA when the drugs were given after the ^{3}H-NA. Thus, the reductions in ^{3}H-NA caused by the drugs were due to blockade of uptake and not to increased release of ^{3}H-NA.

Table 2. *Inhibition of basal gastric acid secretion by Lu* 3–010

Drug	Dose mg/kg, i.p.	Gastric acid secretion m-equiv acid/3 h ± s.e. Exp I	Exp II	Exp III	% Inhibition Exp I	Exp II	Exp III
Water		0·47 ± 0·04	0·44 ± 0·03	0·43 ± 0·07			
Lu 3–010 ..	1·25		0·21 ± 0·03 ($P<0·001$)	0·19 ± 0·03 ($P<0·01$)		51	56
	0·63	0·27 ± 0·03 ($P<0·01$)	0·28 ± 0·05 ($P<0·02$)		43	36	
	0·32	0·40 ± 0·06					
Imipramine ..	5·0		0·20 ± 0·04 ($P<0·001$)			54	
	2·5	0·29 ± 0·05 ($P<0·02$)	0·30 ± 0·02 ($P<0·01$)		38	31	
	1·25	0·37 ± 0·02					
Desipramine ..	2·5			0·15 ± 0·01 ($P<0·001$)			64
	1·25	0·32 ± 0·04 ($P<0·05$)	0·29 ± 0·04 ($P<0·01$)		31	35	
	0·63	0·50 ± 0·06					

Table 2 shows the effects of the drugs on basal gastric acid secretion in the rat. At 1·25 and 0·63 mg/kg, Lu 3–010 inhibited gastric acid secretion 53 and 39%, respectively. Imipramine caused decreases at 5·0 (54%) and 2·5 (34%) mg/kg and the levels declined 64 and 33% after desipramine at 2·5 and 1·25 mg/kg, respectively. Lu 3–010 thus exhibited a higher inhibitory activity than desipramine (2 times) and was even more active (4 times) than imipramine.

In the pylorus-oesophagus-ligated rat, prior administration of Lu 3–010 or desipramine inhibited the stimulation of gastric acid secretion caused by pentagastrin. In animals receiving Lu 3–010, 1·25 mg/kg, the level of gastric acid secretion, was decreased to the level in the control animals; at 0·63 mg/kg, Lu 3–010 caused no significant change (Table 3). Desipramine, 10 mg/kg, prevented the pentagastrin-induced increase and 5·0 mg/kg partially prevented the increase. Lu 3–010 was thus 9 times the more potent.

The effect of Lu 3–010 and imipramine on the multiple stress ulcer is shown in Table 4. Both drugs protected against the development of gastric lesions in a dose-dependent manner. The ED50 ± s.e. was 6·3 ± 1·4 for Lu 3–010, while in the same test the dose of imipramine protecting 50% of the rats was 1·9 ± 1·8.

Results of the 17-h Shay test showed that both Lu 3–010 and imipramine inhibited the ulcer development about 50% at 10 mg/kg and at 20 mg/kg Lu 3–010 gave a 74% inhibition. At 5 mg/kg neither drug had an inhibiting effect.

Table 3. *Inhibition of pentagastrin-induced gastric acid secretion by Lu* 3–010

Drug	Dose mg/kg, i.p.	Gastric acid secretion μ-equiv acid/2 h + s.e. Exp I	Exp II	Exp III	% Inhibition Exp I	Exp II	Exp III
Water + saline ..		18 ± 4	10 ± 3	13 ± 2			
Water + pentagastrin* ..		78 ± 13	54 ± 10	56 ± 8			
Lu 3–010 + pentagastrin*	1·25	23 ± 7 ($P<0·01$)	13 ± 3 ($P<0·01$)		92	93	
	0·63		50 ± 5				
Desipramine + pentagastrin*	10·0	16 ± 4 $P<0·001$)	14 ± 2 ($P<0·01$)	10 ± 4 ($P<0·001$)	103	91	107
	5·0	41 ± 6 ($P<0·05$)	27 ± 1 ($P<0·05$)	26 ± 4 ($P<0·05$)	62	60	70

* Dose, 5 × 1 μg/kg, subcutaneously.

Table 4. *Inhibition by Lu* 3–010 *of the development of gastric lesions caused by stress*

Drug	Dose mg/kg, i.p.	Number of animals with gastric lesions	Inhibition %
Water		17/20	
Lu 3–010.. ..	2·5	7/8	0
	5·0	11/22	41
	10·0	5/14	58
Imipramine ..	2·5	8/21	55
	5·0	6/19	62
	10·0	8/17	45
	20·0	3/17	79

DISCUSSION

Lu 3–010 [3,3-dimethyl-1-(3-methylaminopropyl)-1-phenylphthalan] inhibits the amine transport mechanism of the adrenergic cell membrane in the mouse heart when given intravenously (Waldeck, 1968) and in both the mouse and rat heart when administered intraperitoneally (present findings). In comparison with the known blockers of ^{3}H-NA uptake, imipramine (Axelrod, Hertting & Potter, 1962) and desipramine (Iversen, 1965), Lu 3–010 is about 5 times as potent as imipramine and is comparable in activity to desipramine in inhibiting the ^{3}H-NA uptake into the mouse and rat heart. That desipramine exhibits a higher activity than imipramine in the blockade of ^{3}H-NA uptake agrees with Iversen (1965).

As has been found in the present studies with Lu 3–010, imipramine also inhibits basal (Bonfils, Dubrasquet & Lambling, 1962; Bass & Patterson, 1967; Lippmann, 1969) and pentagrastrin-induced (Lippmann, 1970) gastric acid secretions and restraint-induced ulcer formation (Bonfils, Dubrasquet & others, 1960). Since imipramine reduces gastric acidity and relieves pain in ulcer patients (Varay, Bertheldt & others, 1960), Lu 3–010 may also be of value in these conditions.

It is relevant that catecholamines as well as 5-hydroxytryptamine inhibit gastric acid secretion (Forrest & Code, 1954; Harries, 1956; Haverback, Bogdanski & Hogben, 1958). Entry into the nerve ending, rather than enzymatic destruction, is the main mechanism for the termination of the biological action of noradrenaline

(Whitby, Axelrod & Weil-Malherbe, 1961). The ability of Lu 3–010 to block this uptake of noradrenaline, thereby preventing the inactivation, could thus be of significance in the observed antisecretory activity of this compound.

It is possible that other activities of such drugs are also relevant in relation to their antisecretory actions. Imipramine has been demonstrated to possess anticholinergic activity (Domenjoz & Theobald, 1959), while Lu 3–010 has the advantage of being devoid of anticholinergic activity (Petersen, Lassen & others, 1966).

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The influence of protein binding on the excretion of some sulphanilamidopyrimidines in man

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The metabolism and excretion of sulphadimethoxine, sulphorthodimethoxine, sulphamethomidine, sulphasomidine and sulphadimethoxypyrimidine have been investigated in man. The plasma protein binding data including the percentage bound at various concentrations of the drugs, the number of binding sites (n), the binding capacity of plasma albumin (r_{max}) and the dissociation constant of the sulphonamide-albumin complex (K) for these compounds have been measured by ultrafiltration. The short-acting sulphasomidine is bound to a much lesser extent at the concentrations obtained clinically in the plasma than the long-acting drugs. Sulphasomidine occupies only one binding site on plasma albumin compared with the other sulphonamides investigated which occupy two sites. This may partly account for the rapid excretion of this compound in man. Sulphadimethoxine has two major metabolites, the N^4-acetyl derivative and sulphadimethoxine-N^1-glucuronide. N^1-Acetyl sulphadimethoxine has similar binding constants to the parent compound. However the N^1-glucuronide forms a weak complex with plasma albumin which has a lower binding capacity for this metabolite (r_{max} = 0·58) compared with sulphadimethoxine itself (r_{max} = 1·77). The results are discussed in relation to the excretion of these compounds.

The metabolism and excretion of several clinically important sulphanilamidopyrimidines in man and other species have been investigated.

Sulphadimethoxine (2,4-dimethoxy-6-sulphanilamidopyrimidine, Madribon) (Bridges, Kibby & others, 1968), and sulphamethomidine (4-methoxy-2-methyl-6-sulphanilamidopyrimidine) (Bridges, Walker & Williams, 1969b) are long-acting sulphonamides in man (25% of the oral dose being excreted in the 24 h urine). Sulphorthodimethoxine (4,5-dimethoxy-6-sulphanilamidopyrimidine, Fanasil) (Bridges, Kibby & others, 1969a) and sulphadimethoxypyrimidine (4,6-dimethoxy-2-sulphanilamidopyrimidine, Sulphamoprine) (Walker & Williams, 1969) are very long-acting (6% and 10% of the dose fed excreted in 24 h respectively). Sulphasomidine (2,4-dimethyl-6-sulphanilamidopyrimidine, Elkosin) (Bridges, Walker & Williams, 1969b) is a short-acting sulphonamide in man (72% excreted in the 24 h urine).

The main excretory products of these sulphonamides are the unchanged drug, the N^4-acetyl derivative and the N^1-glucuronide. N^4-Acetyl sulphorthodimethoxine is the major metabolite of this sulphonamide in man (85% of the first 24 h urine) whereas sulphasomidine is excreted mainly unchanged (96% of the first 24 h urine). The N^1-glucuronides of sulphamethomidine and sulphadimethoxine are the major metabolites of these two sulphonamides (68% and 70% respectively of the first 24 h

urine). Sulphadimethoxypyrimidine is excreted in roughly equal amounts as N^1-glucuronide (29%) and unchanged compound (30%) and to a lesser extent as the N^4-acetyl derivative (18% of the first 24 h urine).

The duration of action of these sulphonamides may be related to a number of factors including: rate of absorption and distribution, nature and rate of metabolism, renal excretion and reabsorption, and tissue binding. The plasma protein binding data for these compounds has been measured by ultrafiltration and the results discussed in relation to the urinary excretion data. The protein most generally involved in drug interaction is albumin (Thorp, 1964). Experiments by Anton (1960) and by Newbould & Kilpatrick (1960) have demonstrated the ability of albumin to bind sulphonamides in *in vitro* experiments. Clausen (1966) has shown that although some sulphonamides may bind to other plasma proteins, quantitatively this is only of minor importance. The experiments of Jardetzky & Wade-Jardetzky (1965) using high-resolution nuclear magnetic resonance indicate that the *p*-aminobenzenesulphonamide moiety is the primary and probably the sole binding site in the simpler N^1-substituted derivatives of sulphanilamide.

EXPERIMENTAL

Materials

Sulphadimethoxine m.p. 200–201°, N^4-acetyl sulphadimethoxine m.p. 210–211°, sulphasomidine m.p. 240–241°, sulphorthodimethoxine m.p. 195–197° were gifts from Roche Products Ltd., Welwyn Garden City, Herts. Sulphadimethoxypyrimidine m.p. 179–180° was a gift from Imperial Chemical Industries Ltd., Alderley Park. Sulphamethomidine m.p. 177–178° was a gift from Warner-Lambert, Morris Plains, New Jersey. Sulphadimethoxine N^1-glucuronide (ammonium salt) m.p. 150–160° (decomp.) was synthesized (Bridges, Kibby & Williams, 1965).

Protein binding

Fresh human blood (40 ml) mixed with heparin (0·4 ml or 2000 units; Weddel Pharmaceuticals, London, E.C.1) was centrifuged at 2000 *g* for 10 min. The sulphonamide drug was dissolved in the plasma (20 ml) and 4 ml of 0·1M citrate-0·2M Na_2HPO_4 buffer, pH 7·4 added. The resulting solution (5 ml) was placed in a bag made of Cellophane tubing tied at both ends (15 cm × 3 cm flat width). The bag was placed with a flat surface against a sintered-glass disk (20 mm diam.; porosity 1, Pyrex Co., Sunderland) near the bottom of a polythene centrifuge tube (10 cm × 2·7 cm). The tube was centrifuged at 3000 *g* for 2 h at 18°. The contents of the bag were analysed for total drug (bound and unbound) by adding 20% (w/v) trichloroacetic acid (1 ml) to the plasma (1 ml), then centrifuging at 2000 *g* for 5 min and analysing the supernatant by the Bratton & Marshall (1939) method. The ultrafiltrate below the sintered-glass disk in the polythene tube was analysed for unbound drug by the Bratton & Marshall method.

A graph of r (representing mol of sulphonamide bound per mol of albumin) was plotted against X (representing mol of unbound sulphonamide). The quantity of albumin in human plasma was assumed to be 0·63 mM and the molecular weight of human albumin to be 69 000. At high concentrations of unbound drug the total number of mol of sulphonamide bound per mol of protein (r_{max}) remains constant. This constant was measured at a total sulphonamide concentration in the plasma of 1·6 mM, and it is a measure of the binding capacity of the protein.

For calculations of the binding constant, K, and the number of binding sites, n, the equation of Goldstein (1949) was used in the form:

$$\frac{1}{r} = \frac{K}{n} \cdot \frac{1}{X} + \frac{1}{n}$$

The values of K and n were obtained from the straight line plot of 1/r against 1/X. The slope of the line is K/n and the intercept on the 1/r axis is 1/n. The dissociation constant (K) of the binding reaction between drug and protein is inversely proportional to the strength of binding (Goldstein, 1949).

RESULTS AND DISCUSSION

Bridges & others (1969a, b) have shown that the physical properties of these sulphanilamidopyrimidines and their metabolism affect the rate of urinary excretion. However binding to human plasma proteins also appears to be an important factor in determining their excretion rate. The percentage bound at various concentrations and the binding constants for these sulphonamides can be seen in Table 1.

Table 1. *The binding of some sulphanilamidopyrimidines and their metabolites to human plasma proteins*

Sulphonamide	% bound* at total sulphonamide concentration 0·4 mM	0·8 mM	1·2 mM	K constant mM	r_{max}* at sulphonamide concentration 1·6 mM	Number of binding sites (n)
Sulphasomidine	67	58	44	0·20	1·02	1·1
Sulphamethomidine	90	85	79	0·10	1·73	2·1
Sulphorthodimethoxine ..	96	88	79	0·08	1·91	2·1
Sulphadimethoxypyrimidine	95	80	75	0·25	1·86	2·2
Sulphadimethoxine	92	85	80	0·10	1·77	2·0
N^4-Acetylsulphadimethoxine	90	83	78	0·11	1·80	1·9
Sulphadimethoxine N^1-glucuronide	35	29	23	1·04	0·58	1·0

* The average of three values.

The results show that the short-acting sulphasomidine in man is bound to a much smaller extent at all concentrations measured than the longer-acting sulphonamides. At a concentration of 0·4 mM, which is the plasma level obtained 4 h after an oral dose of this drug (0·05 g/kg) (Prior & Saslaw, 1951), sulphasomidine is only 67% bound, whereas sulphamethomidine, sulphadimethoxine, sulphorthodimethoxine and sulphadimethoxypyrimidine are 90%, 92%, 96% and 95% bound respectively. At concentrations obtained clinically, the long-acting sulphanilamidopyrimidines are highly bound. The percentage of bound drug decreases as the concentration of the sulphonamide increases (Table 1). The r_{max} value for sulphasomidine is less than 60% that of the other drugs having only one binding site instead of two per molecule of albumin. Sulphasomidine is very water soluble and so is rapidly excreted in the urine unchanged. The other sulphonamides being highly bound, less water soluble and more lipid soluble (Bridges & others, 1969a, b) are metabolized before being excreted. However no relation could be found between the charge distribution over the sulphonamide molecule (electronic indices) and the strength of binding of these drugs to plasma albumin.

A comparison between the protein binding data for sulphadimethoxine and the

two major metabolites N^4-acetyl sulphadimethoxine and sulphadimethoxine-N^1-glucuronide is made in Table 1. The N^4-acetyl derivative is bound to approximately the same extent as the parent compound, the K constant and r_{max} are also very similar. Each molecule of albumin can bind either two molecules of sulphadimethoxine or the N^4-acetyl derivative. However sulphadimethoxine is not excreted as rapidly as the N^4-acetyl metabolite because the latter has a much lower lipid-water partition coefficient (Bridges & others, 1969a) and is probably not reabsorbed by the kidney to the same extent. Sulphadimethoxine N^1-glucuronide is not bound to human plasma protein to the same extent as the parent compound (35% compared with 92% at a concentration of 0·4 mM). These differences are reflected in the r_{max} constant (1·77 for sulphadimethoxine and 0·58 mol/mol of albumin for the N^1-glucuronide). Not only does the protein bind less N^1-glucuronide than the parent compound, but the strength of the protein-N^1-glucuronide complex (K = 1·04) is only one tenth that of the protein-sulphadimethoxine complex (K = 0·10). Sulphadimethoxine is therefore poorly excreted as it is lipid soluble, highly and tightly bound at low concentrations (0·4 mM), whereas the N^1-glucuronide, having a low lipid solubility and being poorly and weakly bound, is rapidly excreted.

Conclusion

It is apparent that a small change in the structure of a sulphonamide may alter the binding of that sulphonamide to plasma proteins, i.e., substitution of a methoxyl group for a methyl group of sulphasomidine in position '4' and there is a large increase in the percentage bound. Certain metabolic pathways of sulphonamides alter the binding characteristics. Acetylation of sulphadimethoxine in the N^4 position alters the percentage bound to a minor extent. However the attachment of a glucuronic acid moiety in the N^1 position results in a large reduction in the binding capacity of the protein and a decrease in the strength of the sulphonamide-protein complex.

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The effect of sodium salicylate on the release of acid phosphatase activity from rat liver lysosomes *in vitro*

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Salicylate, in concentrations up to 10mM, does not inhibit the activity of the acid phosphatase liberated from rat liver lysosomes. The release of the enzyme from lysosomal preparations incubated with the drug depends on the pH of the incubation medium, the duration of incubation and the concentration of salicylate. It is concluded that salicylate does not influence lysosomal stability *in vivo*.

The release of lysosomal enzymes may play an important part in several types of cell injury (de Duve, 1964). It has often been stated that one or more phases of either acute or chronic inflammatory reactions may be mediated by the intracellular release of such enzymes. An interesting corollary to this view is that anti-inflammatory drugs may act by interfering with either the release or the activities of the liberated enzymes. Duthie (1963) was the first to suggest this mechanism of action for salicylate and several workers have studied the *in vitro* effects of the drug on lysosomal preparations and enzymes. The results appear to be confusing and contradictory. Salicylate and acetylsalicylate have been reported to decrease the release of enzymes and hence stabilize the lysosomes (Miller & Smith, 1966; Tanaka & Iizuka, 1968), to have no effect (Weissman, 1968; Robinson & Willcox, 1969; Ennis, Granda & Posner, 1968), to increase the liberation of enzymes (Lee & Spencer, 1969; Brown & Schwartz, 1969) and to inhibit the activities of the free enzymes (Anderson, 1968). Differences in experimental conditions may explain, at least in part, these discrepancies and the most important variables seem to be the concentration of the salicylate, the pH and the time of incubation. We have therefore investigated the effects of 0 to 4mM salicylate on the release of lysosomal enzymes over the pH range 5·0 to 7·0 using periods of incubation up to $4\frac{1}{2}$ h.

EXPERIMENTAL

Preparation of lysosomal suspension

Male Wistar rats, weighing between 300 and 500 g, maintained on M.R.C. cube diet No. 41, were killed by cervical fracture. The liver was removed, weighed and minced with scissors in ice-cold 0·25 M sucrose. The sucrose solution was replaced twice to remove as much blood as possible, and the liver homogenized in 10 vol of the sucrose medium using one stroke of a glass homogenizer and a Teflon pestle. The homogenate was centrifuged for 10 min at 3500 *g* to remove nuclei, cell debris and heavy mitochondria. This and subsequent centrifugations were done at 2°. The supernatant was centrifuged for 10 min at 25000 *g* and the lysosomal pellet washed with 5 vol of ice-cold sucrose and finally resuspended in 100 ml of the 0·25 M sucrose (lysosomal suspension).

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Effect of salicylate on activity of free acid phosphatase

The acid phosphatase was liberated from the lysosomal suspension by the addition of sufficient Triton X-100 to give a final concentration of 0·2% (v/v). The membranes and any intact lysosomes were removed by centrifugation for 10 min at 25000 *g* and the supernatant used for the subsequent experiments. The triton X-100 was not removed at this stage because it was found to have no effect on the activity of the acid phosphatase. The reaction mixture contained sodium salicylate, 0–10mM; sufficient sodium chloride to give a final sodium ion concentration of 10 mM and 0·8 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis) in 0·05 M acetate —0·25M sucrose buffer, pH 4·8. The reaction was started by the addition of 3·0 ml of the reaction mixture to 0·1 ml of the lysosomal supernatant, which had been preincubated for 30 min at 37° with either 0·1 ml distilled water or with 0·1 ml sodium salicylate (0–10 mM). The phosphatase assay incubation was performed at 37°, and stopped after 20 min by addition of 1·0 ml ice-cold N NaOH. The *p*-nitrophenol was estimated by measuring the absorption at 410 nm in a Unicam SP 800 spectrophotometer.

Effect of salicylate on release of acid phosphatase activity from the lysosomal suspension

Each reaction mixture contained 1 ml of the lysosomal suspension; 1 ml of a solution containing 0·25 M sucrose and 0·1 M cacodylate buffer at either pH 5·0, 5·5, 6·0 or 7·0; and either sodium salicylate 0 to 4 mM (experimental mixtures) or sufficient sodium chloride to give a final sodium ion concentration equal to that contributed by the sodium salicylate (control mixtures). The mixtures were prepared at 2°, and at zero time were transferred to a waterbath at 37° and incubated with shaking. At appropriate time intervals, up to 4½ h, four experimental and four control mixtures were taken from the incubation bath, cooled on ice and immediately centrifuged at 30 000 *g* for 10 min to remove intact lysosomes and membranes. The supernatants were separated and stored at 0° before being assayed for phosphatase activity. This was performed by taking 0·25 ml aliquots of each supernatant, the reaction being started by the addition of 3·0 ml of a 0·8 mg/ml solution of *p*-nitrophenyl phosphate in 0·25 M sucrose —0·05 M acetate buffer, pH 4·8. The mixtures were incubated for 20 min at 37°, the reaction stopped by adding 1·0 ml of ice-cold NaOH and the absorption at 410 nm measured as described above. Similar experiments were made in which the total acid phosphatase activity of the lysosomal suspension was liberated by 0·2% v/v Triton X-100 and the release of the phosphatase in the presence of salicylate was expressed as a percentage of the values obtained with triton.

RESULTS

Analysis of the results obtained when the enzyme preparation was incubated with salicylate, in concentrations up to 10 mM, and subsequently assayed in the presence of similar concentration of the drug showed no significant ($P = 0{\cdot}02$) inhibition of the free lysosomal acid phosphatase from the control value of 100 ± 2·5%. Experiments in which salicylate was present only during the assay procedure also showed no significant inhibition.

The results of representative experiments concerned with the release of the enzyme from the lysosomal suspensions are given in Table 1. In this Table the values are expressed as percentages of the acid phosphatase activity released by Triton X-100 which has been taken to represent the total enzyme activity originally present in the lysosomes. If the release of acid phosphatase activity is regarded as an index of the

Table 1. *Effect of* 4 mM *salicylate on the release of lysosomal acid phosphatase.* Experimental details were as described in the text. The values, expressed as the percentages of the enzyme activity released by Triton X-100, are given as means of four separate experiments. In the control experiments the mean acid phosphatase activity released by the Triton X-100 was 11·2 μ mol *p*-nitrophenol/mg lysosomal protein in 20 min. The results have been analysed by Students *t*-test, the minimal acceptable level of significance being taken as P = 0·02. (C, control mixture; S, mixture containing 4 mM salicylate).

pH of incubation mixture	Time of incubation (min)									
	30		60		90		180		270	
	C	S	C	S	C	S	C	S	C	S
5·0	5·6	10·0*	22·7	29·6*	41·7	43·2	58·7	58·7	—	—
5·5	1·5	2·3*	7·2	11·4*	22·2	31·6*	55·6	58·1*	—	—
6·0	0·4	0·7*	1·1	1·3*	3·1	4·2*	25·1	32·7*	—	—
7·0	—	—	—	—	2·6	3·0	7·4	8·1	19·8	24·4

* Significant difference between the control and salicylates values

stability of the lysosomes then the pH of the incubation mixture is an important factor. Thus at pH 5·0 approximately 25% of the enzyme activity is released after 1 h incubation whereas at pH 5·5 this is reduced to 7% and is only about 1% at pH 6·0. A long period of incubation is required to cause a release of the enzyme at pH 7·0, e.g. only 7% being liberated after 3 h. The effects of 4 mM salicylate in increasing the release of the enzyme parallel the effects of the pH on the lability of the lysosomal suspensions. The drug is most effective when maximum release of the enzyme is occurring under the corresponding control conditions. This is illustrated in Fig. 1 in which the results for the release of acid phosphatase from the lysosomal suspensions in the presence of 4 mM salicylate are plotted against time of incubation for each of the pH values studied. In

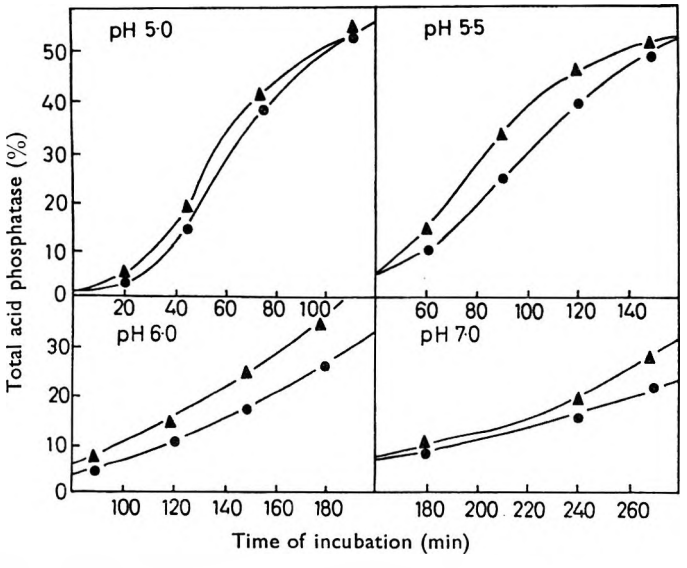

FIG. 1. Effects of salicylate on the release of acid phosphatase from rat liver lysosomes at different pH values. Experimental conditions as in Table 1. ●, control. ▲, 4mM salicylate.

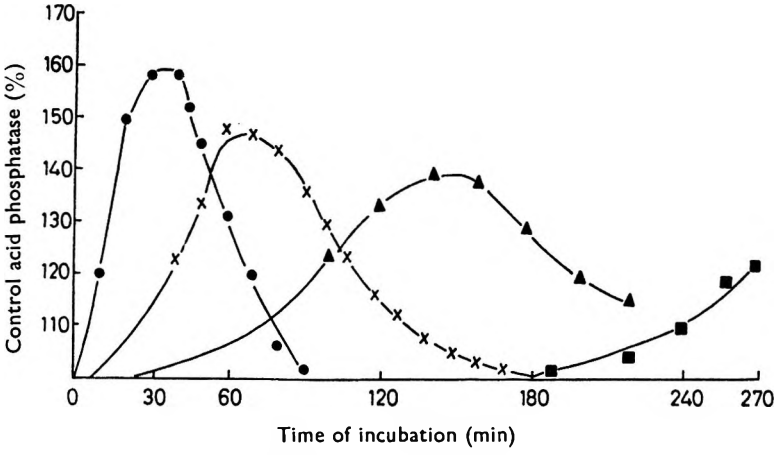

FIG. 2. Effects of varying the pH of the incubation medium on the release of acid phosphatase from rat liver lysosomes in the presence of 4mM salicylate. The data, expressed as percentages of the corresponding control values has been calculated from experimental results. ●, pH 5·0; X, pH 5·5; ▲, pH 6·0 and ■, pH 7·0.

Fig. 2 the results in the presence of salicylate have been expressed as percentages of the corresponding control values and plotted against time of incubation for each pH value. A possible stabilizing action of low concentrations of salicylate could not be studied at neutral pH because little, if any, phosphatase was liberated in several hours. The effects of varying the drug concentration was therefore investigated at pH 6 because the lysosomes were reasonably stable at this pH compared to more acid conditions (Fig. 1). The results in Table 2 show that the only effect of salicylate was to increase the release of acid phosphatase and this became significant at drug concentrations of mM and above.

Table 2. *Effect of varying the salicylate concentration on the release of acid phosphatase from rat liver lysosomes incubated at* pH 6·0 *for* 140 *min.* Experimental details were as described in the text and the results are expressed as in Table 1 except that standard deviations are included.

Salicylate (mM)	Enzyme activity (% of that released by Triton)	*P*
0	10·9 ± 0·7	—
0·5	12·1 ± 1·0	0·1
1·0	12·2 ± 0·3	0·02
2·0	13·0 ± 0·7	0·005
5·0	15·3 ± 0·4	0·001

DISCUSSION

The release of lysosomal enzymes may play an important role in the development of acute and chronic inflammatory reactions (Lack, 1966). It has been suggested that the later stages of rheumatoid joint disease may involve the disruption of intra-articular lysosomes causing the release of hydrolase enzymes which degrade the protein-polysaccharide complexes of cartilage (Ennis & others, 1968). In this situation the proteolytic cathepsins (Barrett, 1969) may be of particular importance but lysosomes

contain many other hydrolases including β-glucuronidase, aryl sulphatases and acid phosphatase (Weissmann, 1967). Acid phosphatase is frequently used as a "marker enzyme" to assess lysosomal damage, both *in vivo* and *in vitro*, because it is localized almost exclusively in the particles, its release parallels that of the other lysosomal hydrolases and its activity may be easily measured. Thus the high activities of acid phosphatase in rheumatoid synovial fluid (Smith & Hamerman, 1962) have been interpreted as evidence for an increased breakdown of lysosomes in the disease. The estimation of the release of the enzyme from lysosomal suspensions is an accepted technique for assessing the integrity of the particles *in vitro*.

Several workers have attempted to explain the actions of anti-inflammatory drugs in terms of interaction between the drugs and lysosomal enzymes. Two possible mechanisms are concerned. The first assumes that the drugs inhibit the activities of the released enzymes. Anderson (1968) reported that aspirin inhibited lysosomal cathepsins and acid phosphatase, the latter enzyme activity being inhibited by about 30% by 1 mM and 67% by 2 mM acetylsalicylate. This has not been the experience of other workers (Tanaka & Iizuka, 1968; Miller & Smith, 1966) and we find salicylate concentrations, up to 10 mM, do not inhibit lysosomal acid phosphatase. Sodium salicylate and aspirin are equally effective in the treatment of acute rheumatic fever and rheumatoid arthritis (Woodbury, 1965) and as anti-inflammatory agents in many acute inflammatory responses in experimental animals (Wilhelmi, Gdynia & Ziel, 1968). There are differences in analgesic potency between aspirin and sodium salicylate (Lim, 1966) and in the antagonism of these drugs to bradykinin-evoked reactions (Collier, 1969) and their effects on the adhesiveness of blood platelets (O'Brien, 1968) but there is no reason to differentiate the two drugs in terms of anti-inflammatory action. Thus any *in vitro* effects of salicylate on lysosomal enzymes should be equivalent to those of acetylsalicylate.

The second mechanism relating the anti-inflammatory activity of salicylate to lysosomal enzymes suggests that the drugs stabilize the lysosomal membrane and hence decrease the liberation of the hydrolases in response to the initial inflammatory insult. Miller & Smith (1966) claimed that acetylsalicylate concentrations of 0·1 mM, and above, decreased the release of acid phosphatase from rat liver lysosomes. The lysosomal suspensions were labilized by incubating at 37° but the pH of the incubation mixtures was not recorded. The importance of this factor was stressed by Tanaka & Iizuki (1968) who reported that variable results were obtained with lysosomal suspensions at neutral pH due to the presence of inorganic salts. Using tris-acetate buffer-sucrose medium at pH 7·4, an ethanolic solution of acetylsalicylate, and a heavy lysosomal fraction, they reported that drug concentrations of 0·5 to 5 mM progressively and significantly decreased the release of acid phosphatase into the medium. In contrast, at pH 5·0, they reported that mM acetylsalicylate caused a slight increase in the release of the enzyme and that this was "drastically accelerated" if the drug was used in a non-buffered medium. The apparent stabilization of lysosomes by acetylsalicylate or salicylate at or about neutral pH has not been confirmed. Weissmann (1968) reported no effect of mM salicylate at pH 6·8, Robinson & Willcox (1969) could not detect any effect of mM acetylsalicylate or salicylate at pH 7·4 and the present results (Table 1) show that 4 mM salicylate does not alter the rate of release of acid phosphatase at pH 7·0 during prolonged incubation. In contrast, the labilizing action of salicylate at pH 5·0 has been observed by several workers with the reservation that

in vitro drug concentrations at mM and above are necessary for the effect to become evident. Thus acetylsalicylate and salicylate concentrations from 0·001 to 1 mM have been reported to have no effect on the release of acid phosphatase and other hydrolases from lysosomal suspensions at pH 5·0 to 6·0 (Ennis & others, 1968) whereas 1–2 mM concentrations of the drug cause an increased release of enzyme under these conditions (Lee & Spencer, 1969; Brown & Schwartz, 1969). The present work shows firstly that decreasing the pH of the incubation mixture increases the lability of the lysosomes when this is assessed in terms of the rate of release of acid phosphatase (Table 1; Fig. 1). Furthermore the effect of salicylate in causing an increased release of the enzyme parallels this lability, becoming more pronounced as the pH of the incubation mixture is reduced (Fig. 2). This effect also increases as the drug concentration is increased (Table 2).

It must be concluded that salicylate does not stabilize lysosomes *in vitro* at neutral pH and that the only interaction between the drug and the subcellular particles is an increased release of acid phosphatase at acid pH. This latter effect is dependent on the pH of the incubation medium, the time of incubation and the concentration of salicylate. A direct interaction between salicylate and either lysosomal membranes or the lysosomal enzymes cannot be the mechanism by which the drugs exert their experimental and clinical anti-inflammatory actions.

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The relative purgative activities of 1,8-dihydroxyanthracene derivatives

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The purgative activities of twelve different 1,8-dihydroxyanthracene derivatives including free anthraquinone, anthrone and dianthrone forms, anthraquinone *O*-glycosides and dianthrone *O*-glycosides were compared with senna pod powder using the production of wet faeces by mice as a criterion of purgation. The higher purgative activity of the dianthrone glycosides was confirmed for the compounds based on rhein. Sennidin (rhein dianthrone) was more active than had previously been reported. These highly active compounds had parallel dose response curves which were not parallel to those of the less active rhein anthrone, rhein, aloe-emodin and chrysophanol. Emodin and chrysazin were inactive in mice. The highly active compounds exerted a high activity during the initial 3 h after dosage while the less active compounds were virtually inactive during this period. Rhein anthrone appeared to act initially like the highly active primary sennosides, sennoside A and sennidin and later as the less active rhein. The results are discussed in relation to the mode of action of orally administered 1,8-dihydroxyanthracene derivatives.

Fairbairn (1949) showed that, in mice, orally administered anthracene derivatives* are highly active as anthrone glycosides, less active as free anthrones and much less active as free anthraquinones. Other workers have confirmed this; anthraquinone glycosides are more active than their corresponding aglycones (Ferguson, 1956); anthrone glycosides, from frangula, are more active than the anthraquinone glycosides (Jørgensen, 1950) and the glycosides glucofrangulin and frangulin are more active than their aglycone emodin (Longo, 1965; Cresseri, Peruto & Longo, 1966). It has been further suggested (Schmid, 1952, 1959; Fairbairn, 1965) that the anthrone form is the active substance *in situ* and that the sugar moiety prevents absorption of the aglycone from the intestine and subsequent detoxication in the liver, or protects it from breakdown in the intestine before it reaches its site of action in the colon and rectum.

To provide additional evidence for these ideas we investigated the relative purgative activities of a larger range of anthracene derivatives than have been studied before at one time.

EXPERIMENTAL

Anthraquinones (I–V)

Aloe-emodin, prepared by the method of Muhtadi (1969), m.p. 222–224° (lit. Fairbairn & Simic, 1963 give 225–226°); λ_{max} 226, 255, 287 and 430 nm (lit. Auterhoff & Scherf, 1960, give 220, 255 and 287 nm).

* The term "anthracene derivatives" refers to those compounds with a nucleus of 1,8-dihydroxyanthraquinone, 1,8-dihydroxyanthrone or dianthrones of the latter. Similarly the terms "anthraquinone", "anthrone" and "dianthrone" all refer to the 1,8-dihydroxy forms.

Rhein, prepared by the method of Oesterle (1902, 1903), m.p. 318–320° (lit. Nawa, Uchibayashi & Matsuoka, 1961, give 321°); λ_{max} 229, 259 and 430 nm (lit. Nawa & others, 1961, give 229 and 258 nm).

Chrysophanol, prepared by the method of Oesterle (1911), m.p. 193–195° (lit. Naylor & Gardner, 1931, give 196°); λ_{max} 229, 255, 289 and 430 nm (lit. Auterhoff & Scherf, 1960, give 222, 251 and 289 nm).

Emodin, obtained in a pure form from another member of the laboratory.

Chrysazin, Bayer Products Ltd.

Anthrones (VI)

Aloe-emodin anthrone, prepared by the method of Rosenthaler (1932), m.p. 196° (lit. Hauser, 1931, gives 195°); λ_{max} 223, 259, 292 and 378 nm (lit. Kinget, 1967, gives λ_{max} 220, 258 and 291 nm).

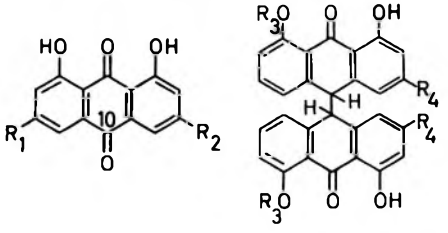
Rhein anthrone, prepared from an impure sample (containing 65% rhein anthrone) by preparative chromatography. Narrow bands of the impure rhein anthrone, dissolved in acetone, were applied to adsorbent layers (0·75 mm) of Kieselgel G (slurries prepared with citrate buffer at pH 6·3) and the plates developed in toluene–methanol (2:1). The band corresponding to the reference rhein anthrone was carefully scraped off and the rhein anthrone eluted from the Kieselgel G with 1% hydrochloric acid in acetone. The eluates from several plates were combined, evaporated to small volume and diluted with water. The precipitate of rhein anthrone was filtered, washed with water, and dried under vacuum at 90°. M.p. 270–300° (decomp.) (lit. Auterhoff & Scherf, 1960, decomp. at 288°); λ_{max} 207, 267, 296 and 372 nm (lit. Lemli, Dequeker & Cuveele, 1963, give λ_{max} 220, 264 and 295 nm).

Dianthrones (VII–VIII)

Aloe-emodin dianthrone, prepared from aloe-emodin anthrone by a modification of the method of Kinget (1967). Aloe-emodin anthrone (100 mg) was dissolved in boiling ethanol (50 ml). To the boiling solution, kept in a dim light, a 1% ethanolic solution of hydrated ferric chloride (10·5 ml) was gradually added over 45 min. The green solution was diluted to 1 litre with water and left overnight at 4°. The precipitate was filtered, washed with water, and dried under vacuum at 90°. The crude aloe-emodin dianthrone was purified by dissolving 30 mg quantities in 5 ml of acetone and passing it through a column of Kieselgel H–Hyflo super cel (1:1) with 1% hydrochloric acid in acetone. The yellow band was eluated and evaporated to small volume and purified by preparative layer chromatography. Bands were applied to absorbent layers of Kieselgel G (0·75 mm) and the plates developed in toluene–cyclohexane–iso-octane-n-propanol (3:5:1:1). The band corresponding to aloe-emodin dianthrone was scraped off and the dianthrone eluted from the adsorbent with the acid acetone solvent. The eluates from several plates were combined, evaporated to small volume and diluted with water. The dianthrone was filtered, washed well with water and dried under vacuum at 90°. M.p. 250–255° (decomp.) (lit. Auterhoff & Scherf, 1960, decomp. 260°); λ_{max} 215, 271 and 370 nm (lit. Kinget, 1967, gives λ_{max} 220, 270 and 365 nm).

Sennidin (Rhein dianthrone) prepared by heating sennoside A (150 mg) in 3N sulphuric acid in a boiling water bath for 30 min. M.p. 330° (decomp.) (lit.

Auterhoff & Scherf, 1960, decomp. above 300°); λ_{max} 215, 270 and 385 nm (lit. Kinget, 1967, gives λ_{max} 220, 275 and 375 nm).



	R_1	R_2			R_3	R_4	
I	H	H	Chrysazin	VII	H	CH_2OH	Aloe-emodin
II	H	Me	Chrysophanol				dianthrone
III	H	CH_2OH	Aloe-emodin	VIII	H	COOH	Sennidin
IV	H	COOH	Rhein				(rhein dianthrone)
V	OH	Me	Emodin				
VI	The anthrones are formed by reduction of the = O on C (10) to H_2			IX	Glucose	COOH	Sennosides A and B

Anthraquinone glycoside

Aloe-emodin 1,8-β-D-*diglucoside*, prepared by the method of Muhtadi & Moss (1969), m.p. 224–226°; λ_{max} 222, 260 and 388 nm.

Dianthrone glycosides (IX)

Crude glycoside concentrate. As no primary sennosides were available an impure sample was prepared from senna pod by the method of Fairbairn, Friedmann & Ryan (1958) which gave a highly water soluble powder containing the calcium salts of the primary sennosides as well as other glycosides (28·1% rhein glycosides, calculated as sennosides A and B, 0·23% of aloe-emodin glycosides, calculated as aloe-emodin monoglucoside and 0·05% free anthraquinones, calculated as rhein).

Sennoside A. The crude glycoside concentrate (4 g) were dissolved in water (40 ml) and the pH of the solution lowered to 2·0–2·5 with a few drops of concentrated hydrochloric acid or the addition of ion-exchange resin [Zeocarb 225 (H^+)]. After standing (60 min) the dark brown precipitate was removed and discarded. The solution was left to stand for 48 h; during this time a yellow precipitate of sennoside A formed. This was filtered, washed with ether followed by water, and dried under vacuum at 90°. (Sennoside B did not precipitate from the solution until after the 48 h period). M.p. 200–220° (decomp.) (lit. Stoll, Becker & Kussmaul, 1949, decomp. 200–240°); λ_{max} 212, 270 and 380 nm (Ref. sennoside A gave λ_{max} 220, 270 and 375 nm).

Standard senna pod

One sample of Alexandrian senna pod in moderately fine powder was used as a standard in all the bioassays. This contained 4·79% rhein glycosides (as sennosides A and B), including primary sennosides, sennosides and rhein glucosides; 0·23% aloe-emodin glycosides (as aloe-emodin monoglucoside) and 0·17% free aglycones (as rhein) including dianthrones.

Biological assay methods

Relative purgative potencies. Dose-response curves and the relative purgative potencies were determined using a modification of the method described in Appendix

III of The Recommended Methods for the Evaluation of Drugs: The Chemical Assay of Senna Fruit and Senna Leaf (1965).

Three separate batches of male albino mice were used in three series of bioassays. 60 or 80 mice divided into groups of 10 were used in each assay. The mice within each group differed by only 1 to 2 g. The anthracene derivatives were orally administered in a dose volume of 1 ml in 2 or 3 dose levels to each group of 10 mice. The range of doses used is listed in Table 1. Senna pod powder was made up as an infusion in hot water. The soluble compounds were dissolved in water and the insoluble compounds were suspended in a 0·2–0·5% w/v solution of compound tragacanth powder. To standardize the conditions compound tragacanth powder was added to the infusion of senna pod powder and to the solutions in similar concentrations. The concentration of the anthracene derivatives in each dose was determined by chemically assaying 1 ml samples. Whenever possible a large number of results for each compound, at different dose levels, was obtained by making several bioassays with each compound. Senna pod powder, in two dose levels was given as a standard in each assay. As only one assay could be made each week the weight of the mice increased (from 20 g to about 50 g) during the series of assays and this was taken into account by expressing the doses as mol/kg of mouse.

Purgative activity was measured by counting the wet faeces produced by each pair of mice 3, 6, 9 and 24 h after administration of the anthracene derivatives. The total number of wet faeces produced by each group of 10 mice (each dose level) over the 24 h period were expressed as "the number of wet faeces/kg of mouse".

The dose levels of the anthracene derivatives were arranged such that the number of wet faeces produced by each group of 10 mice was never greater than 80 to 90 over 24 h. Doses producing this number or less ensured that the size of the wet faeces was constant. Higher doses produced very large wet faeces or such a number that individual ones were difficult to distinguish. When dosed mice were left in the cages for more than the 24 h period no more wet faeces were produced, indicating that the anthracene derivatives were only active during the 24 h period. The results obtained from each batch of mice, for each compound, were plotted as dose response curves. The correlation coefficient and regression coefficient for each compound were calculated if 5 or more results were obtained. If the correlation coefficient was greater than the tabulated value at the appropriate degrees of freedom ($P = 0{\cdot}95$) the regression line was drawn. The parallelity of the different regression lines was determined by comparison of the regression coefficients ($P = 0{\cdot}95$).

Rate of action. The rate of production of wet faeces by the mice after administration of each dose level of each anthracene derivative was determined by expressing the number of wet faeces produced by each group of 10 mice at 3, 6, 9 and 24 h as a percentage of the total number produced over 24 h. The mean of the results for each compound was then found. These ratios were only calculated if the total number of wet faeces produced by each group was greater than 20. The maximum number produced by a group of 10 mice was 90 while the mean was 42.

RESULTS

Chrysazin, emodin, chrysophanol, aloe-emodin, rhein, crude glycoside concentrate and senna pod were tested on the first batch of mice. Chrysazin and emodin produced no wet faeces. The results obtained from the other substances are plotted

Table 1. *Range of doses of anthracene derivatives orally administered to mice*

Anthracene derivative	mg/mouse	mg/kg of mouse	mol/kg of mouse ($\times 10^6$)
Senna pod standard (as sennosides A & B)	0·19– 1·53 (4–32 mg of powder)	4·4– 30·7	4·9– 35·6
Crude glycoside concentrate (as sennosides A & B)	0·28– 1·12 (1·00–2·00 mg of powder)	6·4– 25·0	7·4– 29·0
Sennoside A	0·31– 2·3	6·9– 51·0	8·0– 59·3
Anthraquinone glycoside	2·25– 9·0	45·0–180·0	76·0– 303·0
Dianthrones	0·39– 3·0	7·8– 71·9	14·5– 471·0
Anthrones	1·45– 5·7	29·7–124·0	105·0– 457·0
Anthraquinones	1·8 –11·6	40·0–300·0	115·0–1,111·0

All the anthracene derivatives were given in a dose volume of 1 ml per mouse.

as dose response curves in Fig. 1A. The crude glycoside concentrate was of equal activity to the senna pod standard (both calculated as sennosides A and B) while the anthraquinones were much less active and have parallel dose response curves which were not parallel to that of senna pod.

Sennoside A, sennidin, rhein anthrone, rhein and senna pod, were tested on the second batch of mice; the dose response curves are plotted in Fig. 1B. The dose

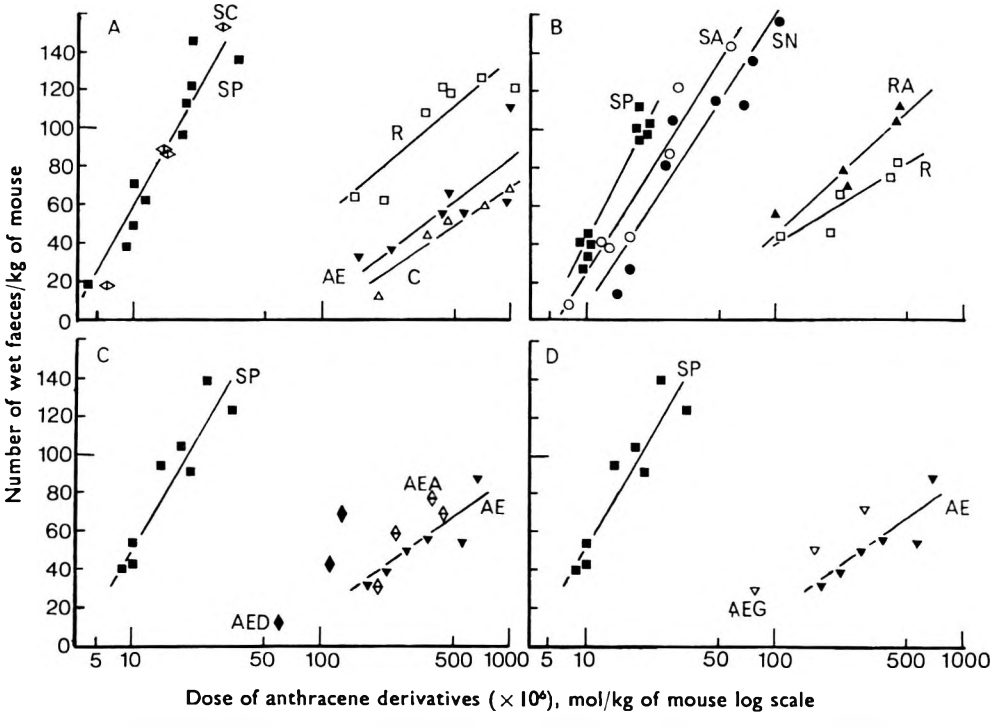


FIG. 1. Log dose response curves of anthracene derivatives tested on mice. A, first batch of mice; B, second batch of mice; C and D, third batch of mice with the same results from senna pod and aloe-emodin plotted in both for convenience. Regression lines are drawn in where there are five or more results. (AE ▼) aloe-emodin, (AEA ⟠) aloe-emodin anthrone, (AED ◆) aloe-emodin dianthrone, (AEG ▽) aloe-emodin diglucoside, (C △) chrysophanol, (R □) rhein, (RA ▲) rhein anthrone, (SP ■) senna pod, as sennosides A & B, (SC ⟠) crude glycoside concentrate, as sennosides A & B, (SA ○) sennoside A, (SN ●) sennidin.

response curves of sennoside A, sennidin and the senna pod standard (latter chemically assayed as sennosides A and B) were all parallel and sennoside A was 68% as active as senna pod and sennidin 53% as active. Rhein anthrone and rhein were much less active and had dose response curves which are neither parallel to the latter, more active compounds, nor to each other.

Aloe-emodin diglucoside, aloe-emodin dianthrone, aloe-emodin anthrone, aloe-emodin and senna pod, were tested on the third batch of mice. The results are plotted in Fig. 1C and D, with the same results of senna pod and aloe-emodin in each Figure. All these compounds based on aloe-emodin were far less active than the senna pod standard. Aloe-emodin dianthrone and diglucoside were only slightly more active than aloe-emodin. Unfortunately, not enough results were obtained to calculate regression lines for the former two compounds. Aloe-emodin anthrone had a similar activity to aloe-emodin but again no regression line could be calculated. However, the results for aloe-emodin anthrone, dianthrone and diglucoside all appear to lie on a slope more similar to that of aloe-emodin than senna pod.

Consistency of responses and relative potencies. The many experiments necessary involved using three successive batches of mice over two years. As already indicated the increase in weight of each mouse was compensated for by expressing the doses

Table 2. *Consistency of responses shown by the three different batches of mice after oral administration of senna pod*

Batch of mice adminstered with the standard senna pod	Correlation coefficient (r)	Tabulated r for appropriate ϕ ($P=0{\cdot}95$)	Regression coefficient†	*Dose to produce 70 wet faeces/kg mol/kg $\times 10^6$
1	0·94	0·58	178·2	11·5
2	0·96	0·67	191·3	13·5
3	0·93	0·71	169·5	14·4

* The doses are obtained from the regression lines plotted on the graphs in Fig. 2.
† The regression coefficients were shown to be not significantly different.
ϕ Degrees of freedom.

Table 3. *Relative potencies of anthracene derivatives compared to standard senna pod*

Anthracene derivative	Relative potency (in mol) (at dose * to produce 70 wet faeces/kg of mouse)	
Senna pod (as sennosides A & B)	100	
Crude glycoside concentrate (as sennosides A & B)	100	
Sennoside A	68	Parallel log dose/ response curves.
Sennidin (Rhein dianthrone)	53	
Rhein anthrone	7	
Rhein	5	
Aloe-emodin diglucoside	4 (approx.)	Parallel log dose/
Aloe-emodin dianthrone	4 (approx.)	response curves,
Aloe-emodin anthrone	2 (approx.)	(not parallel to
Aloe-emodin	2	above).
Chrysophanol	2	
Chrysazin	Inactive	
Emodin	Inactive	

* Doses obtained from the regression lines wherever possible, plotted on the graphs illustrated in Fig. 1.

and responses as the weight, or number, per kg of mouse. Other variables were accounted for by using a standard sample of senna pod in each assay. The results in Table 2 show that there was a remarkable consistency in the responses of the different batches of mice to the standard senna pod. The regression coefficients were compared and found not to be significantly different, i.e., the regression lines of the dose response curves are parallel for all three batches of mice. The relative potencies of senna pod between the three batches was also similar. Rhein and aloe-emodin were the only other compounds tested on more than one batch of mice; the regression coefficients of these compounds were found to be similar in all three batches of mice, the relative potencies varied more than those found for senna pod. Thus the senna pod and two anthraquinones had a fairly consistent action on the different batches of mice. Although the dose response curves of the less active compounds were not parallel to those of the highly active sennoside A, sennidin and senna pod, the difference in potency of the two groups was such that the relative potencies of all the compounds tested could be compared at the dose level to produce 70 wet faeces/kg of mouse (the average of the number of wet faeces produced by the highest and lowest doses of senna pod) and related to senna pod as a standard. The results are shown in Table 3.

Comparison of the rates of production of wet faeces. The results are illustrated in Fig. 2. It is seen that they fall into two groups. Senna pod, senna pod concentrate, sennoside A, sennidin and rhein anthrone had their greatest activity, producing about 40% of the total number of wet faeces, in the initial 3 h after dosage while rhein, aloe-emodin and the other compounds based on aloe-emodin had only produced about 8% of the total number during this time. After 9 h the former group of compounds had produced about 90% of the total number of wet faeces while the latter group had produced only 67%.

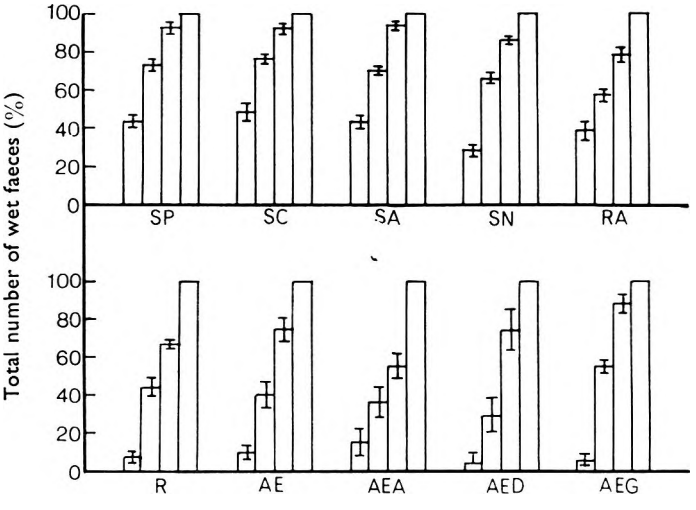


FIG. 2. Rate of production of wet faeces by mice after oral administration of anthracene derivatives. The columns represent the numbers of wet faeces, as a percentage of the total number produced over 24 h, produced during the 3, 6, 9 and 24 h periods after administration of the anthracene derivatives. The results are the mean of the results obtained from each dose level and the vertical lines represent the standard error of the mean. Key, as in Fig. 1.

DISCUSSION

Our results confirm the claims made by previous workers that glycosides are more active than aglycones and that anthrones are more active (only for rhein anthrone, not aloe-emodin anthrone) than anthraquinones as can be seen from Fig. 1 and Table 3. The present work respresents a more extensive and intensive study than has been done previously. The calculated correlation coefficients were greater than the tabulated values for those compounds from which five or more results were obtained showing that the logarithm of the dose was linearly related to the response for these compounds. This, together with the consistency of the results obtained from senna pod between the three batches of mice (Table 2) establishes that the biological assay method was reliable.

The dose-response curves of the active anthracene derivatives are parallel to each other but not to the low activity derivatives which are also parallel to each other (Fig. 1). A further difference is that the active group showed a high purgative activity during the first 3 h whereas the less active group showed little purgative activity initially but a relatively higher activity during the 9–24 h period (Fig. 2). This discrepancy in the parallelity of the dose response curves show that only the highly active compounds can be assayed using a 4-point assay method with senna pod as a standard. In this present series of assays an approximate estimate of the relative potencies between senna pod and the much less active aglycones was made by a comparison of the distances between the log-dose response curves at the dose levels to produce 70 wet faeces/kg. Although this estimate is only valid at this dose level it does illustrate the great difference in purgative activity between the two groups of compounds.

Sennoside A was only 68% as active as senna pod (chemically assayed as sennosides A and B), a result close to that given by Fairbairn (1965) who found that the sennoside content of the pod accounted only for 60% of the total activity. This was attributed to the presence of highly active primary glycosides of the sennosides and since we found that the pod and crude glycoside concentrate were equipotent and their log-dose response curves were parallel to that of sennoside A and sennidin (rhein dianthrone) it seems that the primary sennosides act similarly to the "secondary" sennosides and sennidin. A possible explanation of the differences in potency is suggested earlier (p. 584); that the sugar moiety prevents absorption of the glycosides from the alimentary canal; the more soluble the glycosides, the more will be retained in the lumen. Furthermore the sugars "protect" the active anthrone moiety from oxidation in the relatively alkaline conditions of the small intestine.

The present work has revealed some exceptions to the above suggestions. The aglycone sennidin (rhein dianthrone) was significantly more active than had previously been thought (Fairbairn, 1949). Table 3 shows that it has an activity of about 80% of sennoside A with a parallel dose-response curve. In contrast, rhein anthrone had only about 10% of the activity of sennoside A and the dose response curves are not parallel. This suggests that the dianthrone structure itself is an important factor in protecting the anthrone form from oxidation *en route*. Rhein anthrone, although of relatively low activity (probably because it is readily oxidized in the slightly alkaline conditions of the small intestine), shows high initial activity like that of the active glycosides (Fig. 2) and its dose response curve is not parallel

to either the highly active or less active groups (such as its oxidized form rhein) which indicates that some of it may reach the site of action in the large intestine in the reduced form in the first few hours after dosage. The other aglycones however have a low initial activity and a higher activity during the 9–24 h period, which may suggest that they take longer to reach the large intestine than the more soluble active compounds, or that the presence of larger amounts in the large intestine is necessary for purgation. Thus, these various suggestions may be reasons for the difference in parallelity of the dose response curves. The highly active compounds act in the anthrone form, while the less active compounds act, in a different manner, in the quinone form. The results obtained from the derivatives based on aloe-emodin (Fig. 1 and Table 3) also suggest some modification of the above generalizations. Although formation of the dianthrone and diglucoside leads to an increase in activity, the amount of increase is markedly less than that with the corresponding rhein series and aloe-emodin anthrone has the same activity as aloe-emodin. The slopes of the dose-response curves also appear to differ for the dianthrone derivatives of rhein. Moreover the relative potencies in the aloe-emodin series are much less than in the rhein series, and they have a lower initial activity. The increased activity of the rhein series may be due to the presence of the carboxylic group in rhein, acting directly because of its acidity or by increasing the solubility of the aglycone.

For high purgative activity we therefore suggest the following factors:

(i) Prevention of absorption from the alimentary canal *en route*. This is effected by the presence of sugars increasing water solubility. High molecular weight may also be a factor, since the active substances have molecular weights varying from about 560 to 1200 whereas the less active are about 270; this would explain why rhein dianthrone, though not very water soluble, is of high activity.

(ii) Prevention of oxidation of the active anthrone to the inactive quinone form. This again is effected by the presence of sugars or by a dianthrone structure.

(iii) Water solubility of the *aglycone*, so that it is in solution at its probable site of action in the large intestine.

Acknowledgements

We wish to thank Westminster Laboratories Ltd. for providing the senna pod powder, crude glycoside concentrate and crude rhein anthrone; Dr. F. J. Muhtadi for a sample of emodin; Mr. J. Davey for invaluable help with the bioassays; The Science Research Council for a maintenance grant for one of us (M.J.R.M.). This work forms part of a thesis presented by one of us (M.J.R.M.) for the degree of Ph.D., University of London.

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Moisture uptake and tensile strength of bulk solids

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An apparatus is described in which bulk solid handling properties can be examined over a wide range of humidities. It is shown that constant temperature and relative humidity can be maintained. The apparatus can also be used to study the effect of cycles of humidity changes. The rate of moisture uptake by loosely packed and tapped beds of six pharmaceutical materials was found to be dependent upon bed depth but independent of bulk density. The tensile strengths of the bulk solids changed when they were subjected to high relative humidities.

The presence of moisture in bulk solids can seriously impair the flow properties of materials and thus cause non-uniform dosage in preparations or loss of efficiency in processing. It may, however, be used to advantage in granulation.

The strengths of powder beds containing excess moisture have been measured using a tilting table split plate apparatus (Eisner, Fogg & Taylor, 1960; Shotton & Harb, 1966). More recently, measurements of the strength of moist agglomerates and compressed beds of bulk solids have shown that significant changes in tensile strength can occur according to the physical location of moisture within the specimens (Rumpf, 1958; Smalley & Smalley, 1964; Pietsch, 1967; 1968; Pietsch, Hoffman & Rumpf, 1969); Griffiths (1969) has reviewed the effects of moisture in tablet manufacture.

The application of shear and tensile strength determination to problems of bulk solid flow behaviour permits a complete description of powder behaviour in terms of certain derived parameters (Jenike, 1961; Williams, 1965; Farley & Valentin, 1967–68). These investigations show that particle size, size distribution and shape affect the cohesion and tensile strength of bulk solids (Cheng, 1968) but in all cases the moisture content was not varied.

It would therefore be desirable to investigate the changes in tensile strength that occur when a dry bulk solid is exposed to a humid environment.

It is not convenient to use conventional humidity cabinets for this purpose since transference from high relative humidities to measuring apparatus in laboratory environments leads to severe errors due to loss of moisture by evaporation and desorption. Therefore an apparatus was constructed so that all measurement could be made within a controlled environment.

The tensile strength of bulk solids at different humidities depends, amongst other variables, on the rate of moisture uptake and loss by the powder bed. This, in turn, may be expected to vary with the state of packing and depth of the beds.

Furthermore, caking may occur with some material when moisture is lost. Consequently the apparatus was designed to examine the effects of cyclic variations in humidity. In the first instance, a range of materials of pharmaceutical interest has been studied to indicate the gross effects that may occur.

EXPERIMENTAL

Description of the apparatus

The apparatus (shown diagrammatically in Fig. 1) consists of a stainless steel chamber divided into three gloved compartments through which humidified air is circulated at a constant temperature. The valves, $V_1 \rightarrow V_{10}$ are arranged so that air may be passed through any of the three compartments and through one or both of the humidifiers H_1 H_2.

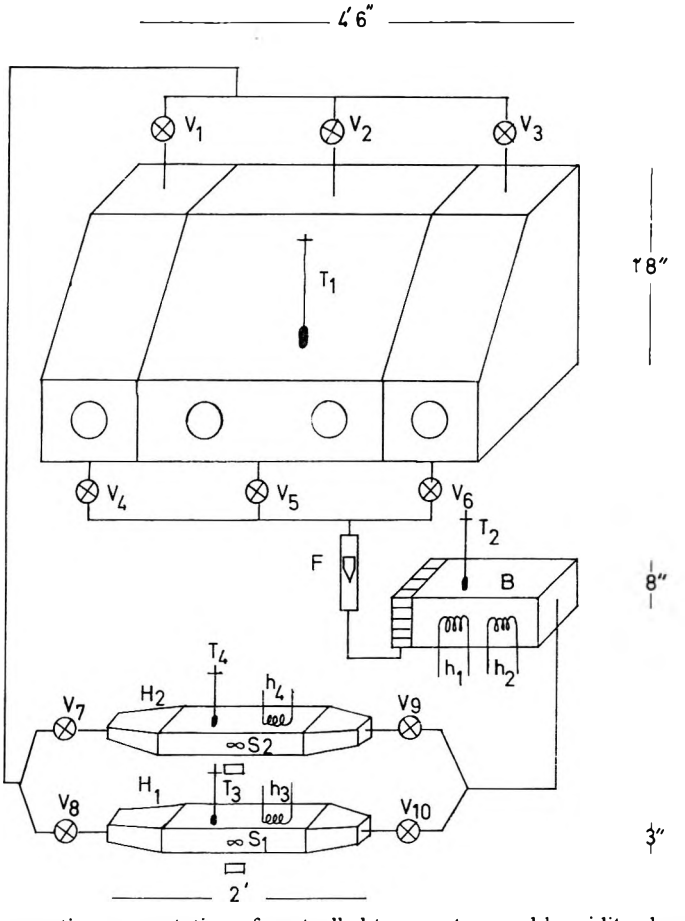

FIG. 1. Diagrammatic representation of controlled temperature and humidity chamber. B, fan box; F, flowmeter; H_1H_2, humidifiers; h_1–h_4, heaters; S_1S_2, mechanically stirred salt solutions; T_1–T_4, control thermometers; V_1–V_{10}, air valves.

The working area has three separate sloping front panels of double glazed $\frac{1}{4}$ inch thick Perspex bolted onto a rubber coated foam gasket. Internally, the three compartments are interconnected by means of detachable doors operated from the end compartments. Access to the chamber for the introduction or removal of apparatus and samples, is achieved by sealing off one end compartment and opening only this to the room.

Air enters each compartment by a 2 inch diameter orifice mid-way along the bottom of the rear wall and is dispersed by a detachable raised floor of stainless

steel. This floor consists of a series of trays perforated at regular intervals with $\frac{1}{2}$ inch diameter holes. The tray nearest the inlet port is only partially drilled to prevent channelling of the inlet air before dispersal.

After passing up the chamber, the air leaves via a central ceiling orifice baffled as a further prevention against channelling.

The temperature of the chamber and the air is maintained constant by an adjustable mercury contact thermometer, T_1, situated in the centre compartment (i.e. the one which is always in use). This controls a 200 W mica "black body" heating element h_1 mounted together with a similar but manually operated heater h_2 for initial warming up. Details of the electrical controls are presented in Fig. 2.

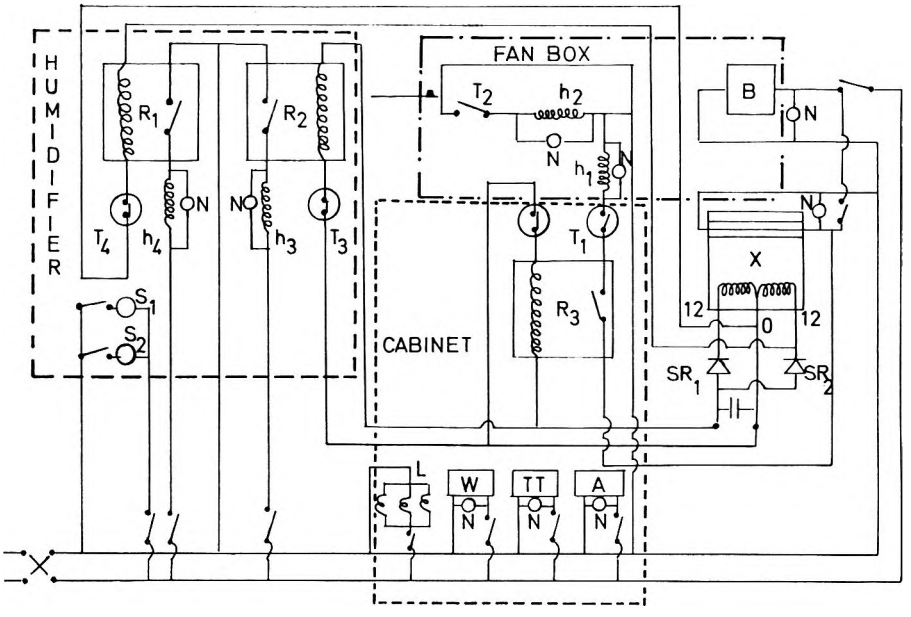

FIG. 2. Circuit diagram of controlled temperature and humidity chamber. Symbols as Fig. 1 plus A, hygrometer; L, lights; N, indicating neons; R_1–R_3, relays; SR_1 SR_2, silicon rectifiers; TT, tensile tester; W, balance; X, transformer.

These heaters are situated in a chamber, B, containing a centrifugal blower (1/30 H.P. shaded pole, ventilated motor with $4\frac{5}{8}$ inch diameter $2\frac{3}{8}$ inch width impeller operating at 2500 rev/min) and excess temperature rises during boost heating are controlled by a bimetallic strip thermostat, T_2, set to open at 70°. This thermostat, which is wired in series with the main and boost heaters, enables the apparatus to be left unattended with safety in case of instrument failure.

Constant humidity is achieved by circulating the air over magnetically stirred saturated salt solutions, S_1 S_2, containing excess undissolved salt. The humidifiers, H_1 H_2, are designed to profile the air flow such that a large evaporative surface is presented to a narrow jet of air.

Establishment of the desired relative humidity may be prolonged if the latent heat of evaporation is not externally supplied since the solutions will remove sensible heat from the circulating air (Garton, 1948). The solutions in the humidifier trays

are therefore maintained at a constant temperature by 100 W pencil-type immersion heaters h_3, h_4 controlled by narrow range adjustable mercury contact thermometers T_3, T_4.

Choice of the salt solution is made by reference to the literature (Wexler, 1965; Young, 1967) from which it can be seen that relative humidities over the range 20–90% may be obtained. Precise adjustment of relative humidity is possible by accurate control of the temperature of the saturated salt solutions.

Using different salt solutions in each humidifier consecutively, changes in relative humidity can be effected, thus permitting a cycle of changes to be made. Reduction in humidity can be rapidly produced by the use of desiccants.

All connections of air supply are by 2 inch diameter plastic flexible hosing and 1½ inch diameter polypropylene manifolds. The air flow rate is measured by using a suspended float-type flow meter, F (range 20–200 litre air/min at 15° and 760 mm Hg) and is controlled by precision unplasticized PVC gate or aluminium butterfly valves. Pressure within the circuit is monitored from the centre compartment by means of a U-tube manometer.

Heat losses are kept minimal by the liberal use of expanded polystyrene and hair felt lagging.

Results of apparatus behaviour

Some common causes of error during the maintenance of atmospheres of known temperature and humidity are listed by Garton (1948). These include the non-uniformity of temperature and partial water vapour pressure, the latter usually arising from condensation, inadequate air mixing or leakage from the enclosure, or both.

The apparatus described above is designed to minimize these problems and has been used successfully at temperatures between 22° and 28°. However, to avoid the influence of high laboratory temperatures, a working temperature of 28° was chosen. All subsequent reference to relative humidity values, therefore, applies to this temperature.

Starting at an ambient temperature of 23° the air reached working temperature within 1 h. Subsequently, 24 h was allowed as a cabinet temperature equilibration period. By careful adjustment of the inlet valves a temperature of $\pm 0{\cdot}5°$ throughout the chamber was obtained.

The lagging proved efficient against temperature fluctuation and only minute condensation on the Perspex front plates was evident at relative humidities approaching saturation.

By inserting the flowmeter at strategic positions in the circuit and closing selected valves the presence of leaks could be detected and corrected.

Investigation of the air flow rates with different compartments sealed off showed that the chamber atmosphere was completely changed at least once every 2 min, varying from 1·2 min for a complete air change of the centre compartment only to 1·9 min for all three compartments. Times much below these would lead to problems of dispersal of powder specimens in the apparatus.

Fig. 3 shows the humidification of the chambers using potassium chloride as the salt in both humidifiers. From this it can be seen that satisfactory equilibrium conditions of relative humidity were achieved in less than 1 h. Similar results were

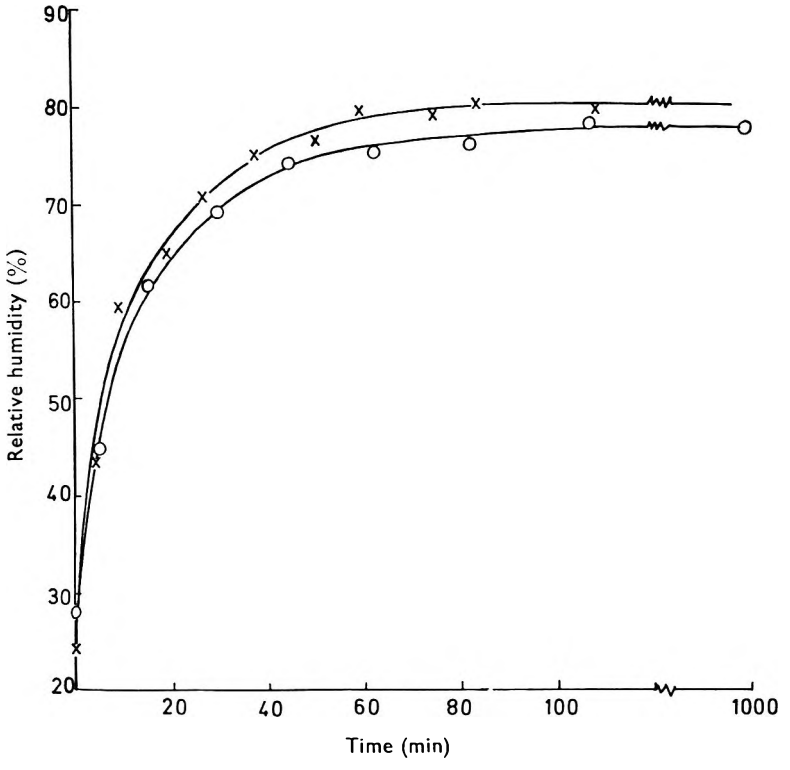

FIG. 3. Humidification of the chamber using saturated potassium chloride solution. ○ two humidifiers T_1, 28° T_3, T_4, 29° × one humidifier T_1, 28° T_3, 30·5°C.

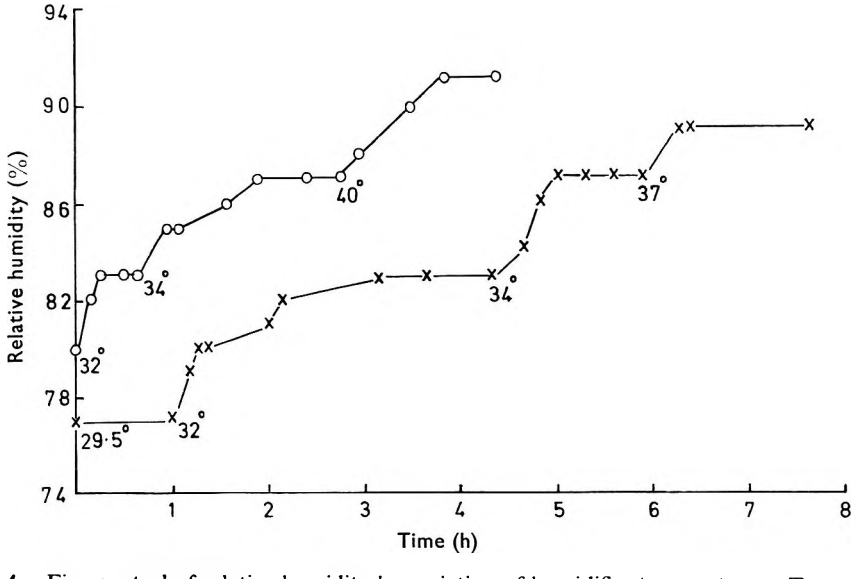

FIG. 4. Fine control of relative humidity by variation of humidifier temperature. Temperature of T_3 adjusted at points indicated. Initial T_3 temperature ○, 32°; ×, 29·5°.

obtained using other salt solutions giving relative humidities of 28% with potassium acetate and 80% with ammonium dihydrogen orthophosphate and relative humidity variation throughout the working area was found to be $\pm 1{\cdot}5\%$.

The variation in relative humidity that was produced on controlled increase of the temperature of the humidifier solution is shown in Fig. 4. An increase of 2–4% was achieved by a 2 or 3° change in temperature. Any particular relative humidity obtained in this way could be maintained with the same accuracy as shown above.

All these humidity changes were followed by means of an aspirated wet and dry bulb thermometer of the Assmann type (accuracy $\pm 1\%$ RH) and continuously monitored using a recording human hair thermohygrograph (accuracy $\pm 5\%$ RH). Temperature constancy was checked by means of 15 mercury-in-glass thermometers.

Reduction in humidity was achieved by the use of 500 g of dried coarse silica gel in each humidifier to 10% relative humidity, below which phosphorous pentoxide impregnated onto an inert matrix proved effective. Table 1 shows that the rate of

Table 1. *Effect of time previously spent at elevated relative humidities on subsequent dehumidification process*

Time previously spent at elevated relative humidity	Dehumidification produced by 500 g dried silica gel in each humidifier in the first h	Subsequent time required for RH to reach 14% (silica gel replaced every 6 h)
1 h at 80%	80% to 28%	—
42 h at 78–91%	78% to 66%	12 h
89 h at 76–89%	77% to 66%	20 h

fall in humidity is determined by the length of time over which the high relative humidity had been previously maintained. This is due to adsorbent and absorbent effects associated with the plastic connection hoses which increased the moisture load of the system. At a cabinet relative humidity of 80% and a laboratory relative humidity of 51%, opening the isolated end compartment, by removing a glove for 10 min, was found to lower the relative humidity by 3% when all three compartments were re-connected to circulating air. Complete recovery to equilibrium relative humidity was achieved after 45 min. Removal of a Perspex front for 5 min produced a similar fall requiring 1 h for complete recovery.

Table 2. *Physical characteristics of materials used*

Material	Shape (Elongation ratio)	Particle density g ml^{-1} 25° Immersion fluid		Bulk density *g ml^{-1}	
		Toluene	Liquid paraffin	Poured	Tapped
Calcium phosphate ..	1·51	3·00	2·91	0·29	0·46
Lactose	1·82	1·55	1·55	0·65	0·80
Potato starch	1·25	1·51	1·51	0·40	0·58
Sodium chloride	1·09	2·16	2·14	1·21	1·36
Potassium chloride ..	1·22	1·96	1·97	1·10	1·21
Sucrose	1·15	1·59	1·59	0·87	1·00

* Bulk densities determined using British Standard (1948) method.

Investigation of bulk solid properties—Materials and Methods

Six bulk solids, differing in both physical and chemical properties, were sampled by chute splitting and dried in thin layers, before investigation, by heating at controlled temperatures in a hot oven for 48 h. The samples were transferred directly to the apparatus maintained at a relative humidity below 25% before humidification. The characteristics of the samples are presented in Tables 2 and 3.

Table 3. *Size characteristics of materials used*

	Size distribution Cumulative percentage greater than stated size * Volume surface diameter (μm)				
Material	80	60	40	20	10
Calcium phosphate ..	—	2	7	22	50
Lactose	2	4	29	69	90
Potato starch	2	15	60	97	99

	Size range†	Arithmetic mean sieve diameter (μm)
Sodium chloride ..	178–250	214
Potassium chloride ..	125–250	187
Sucrose	500–699	600

* Determined using Coulter Counter Model B.
† Prepared by sieving.

In order to investigate some of the factors influencing moisture uptake, representative samples of the bulk solids were packed into Petri dishes and beakers having internal diameters of approximately 50 mm. The beds were prepared by dredging and tapping such that variations in bed depth and bulk density were obtained. Once prepared these samples were left undisturbed during the subsequent humidification period. Material to be used later for tensile strength determinations was exposed in thinly dredged layers on 4 × 2½ × ½ inch aluminium foil trays. Preliminary work had shown that provided the bed depth was of only a few particles thick, moisture distribution throughout the beds was reasonably uniform. As an additional safeguard, material reserved for tensile strength determinations was periodically disturbed by stirring. The apparatus used to determine tensile strength consisted of a horizontal split cell unit similar to that described by Ashton, Farley & Valentin (1964) and the beds were tested immediately after packing into the cell.

Weight increases were determined using a top pan balance (readability 0·001 g precision 0·0005 g) and subsequently checked by determining the weight loss on drying at temperatures which caused no chemical breakdown of the material. These temperatures were selected by reference to thermogravimetric analyses on humidified samples and the results expressed as % weight increase–dry basis.

During the experiments the only attention that the cabinet required was maintenance of the volume of the saturated solutions every two or three days.

RESULTS

Fig. 5 illustrates the effect of bed depth on the weight increase of potato starch, packed to a similar initial bulk density, when exposed to a relative humidity of 85%. It can be seen that the initial establishment of equilibrium humidity conditions is

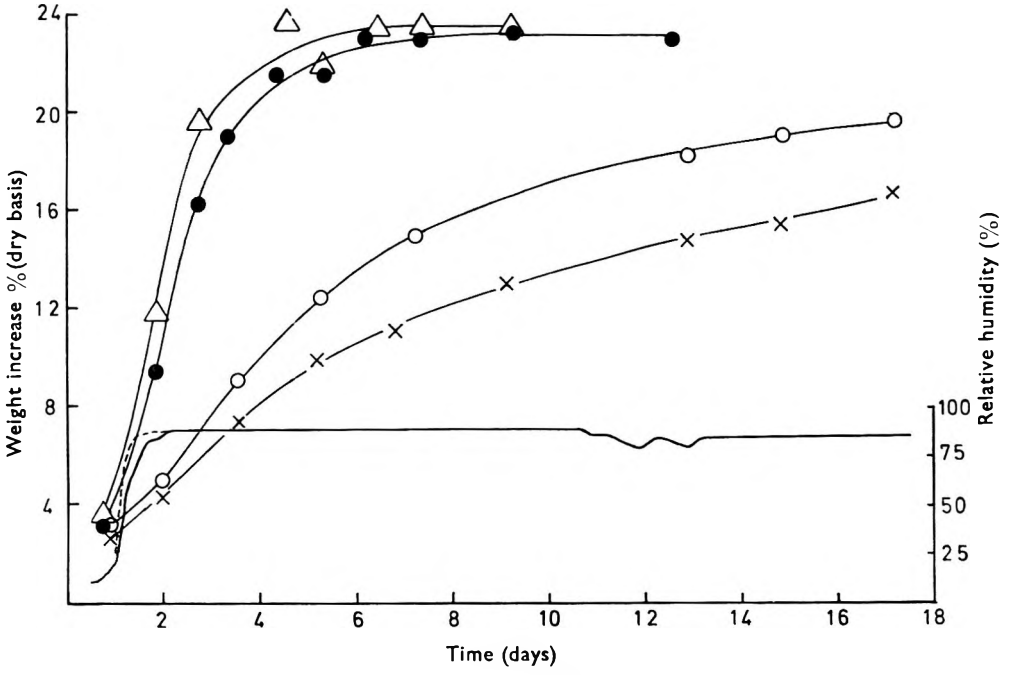

FIG. 5. The effect of bed depth on the moisture uptake of potato starch at similar initial bulk densities. Bulk densities (g ml^{-1}) × 0·918; ○, 0·946; ●, 0·990; △, 0·95. Bed depths (cm) × 4·5. ○, 2·8; ●, 1·2; △, 0·55. Broken line—Cabinet humidification without material present.

retarded. This is due to the excess of material (2 kg) present during testing. It is clear, however, that bed depth significantly influences the uptake of moisture and that equilibrium moisture contents were achieved in a reasonably short period only when thin beds of bulk solid were used.

Similar curves were obtained for lactose and calcium phosphate but in these cases the relative humidity achieved equilibrium in a much shorter time. Equilibrium

Table 4. *Equilibrium moisture contents (EMC) at elevated relative humidities*

Material	Equilibrium R.H.	Total No. of Samples	Mean EMC % w/w loss on drying dry basis	Standard deviation
Calcium phosphate ..	87%	10	1·79	0·08
Lactose..	89%	11	5·27	0·17
Potato starch	85%	14	23·24	0·40

moisture contents are presented in Table 4. The increase in weight during humidification shown in Fig. 5 was not observed with alkali halides and sucrose. Fig. 6, which shows the effect produced when sodium chloride is humidified, indicates that weight increase occurs only above certain critical relative humidities. Again, the effect of bed depth is evident but in these cases no equilibrium moisture content is reached. The results for potassium chloride and sucrose were similar.

Only small variations in weight increase were observed at different states of packing. Fig. 7 shows typical results for filled containers at low bed depth. The

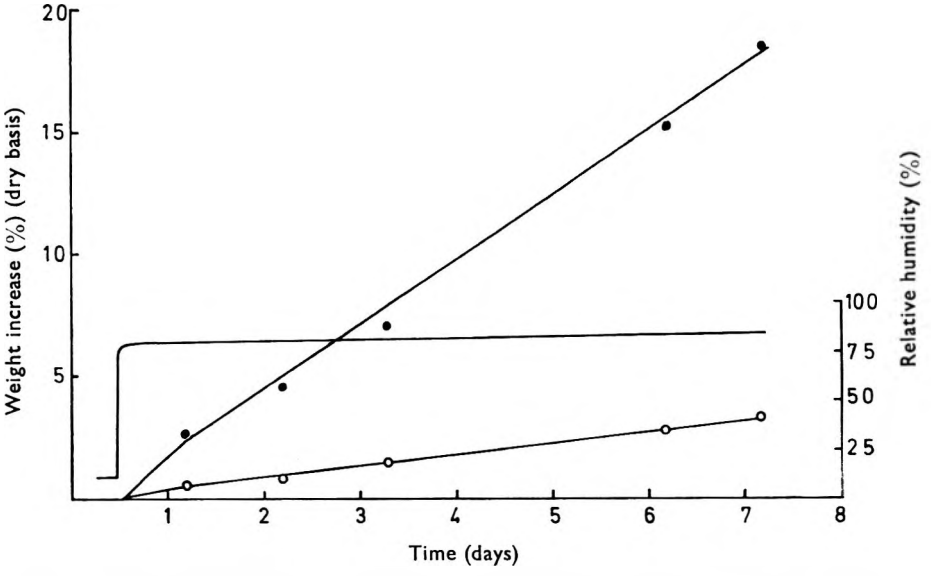

FIG. 6. The effect of bed depth on the moisture uptake of sodium chloride at similar initial bulk densities. Bulk densities (g ml^{-1}) ○, 1·213; ●, 1·260. Bed depth (cm) ○, 2·3; ●, 1·2.

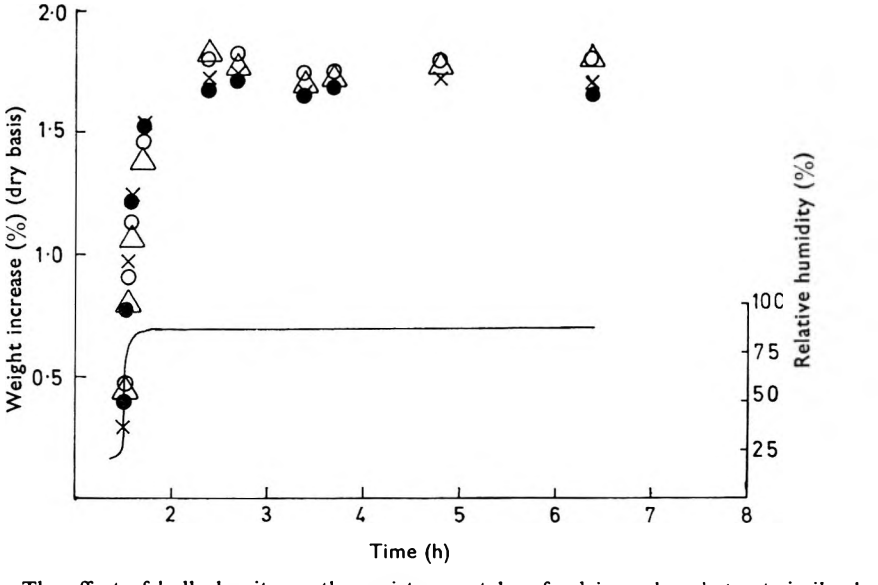

FIG. 7. The effect of bulk density on the moisture uptake of calcium phosphate at similar bed depths. Bulk densities (g ml^{-1}) ●, 0·160; ×, 0·184; ○, 0·218; △, 0·312. Bed depth (cm), ●, 1·15; ×, 1·20; ○, 1·15; △, 1·15.

bulk densities for this material (calcium phosphate) varied from the most open state of packing, produced on dredging, to a high density produced by tapping the material in the final container without a normal consolidating load. The tensile strengths of samples at various relative humidities and moisture contents are shown in Figs 8 and 9. The results are presented as tensile strength versus packing

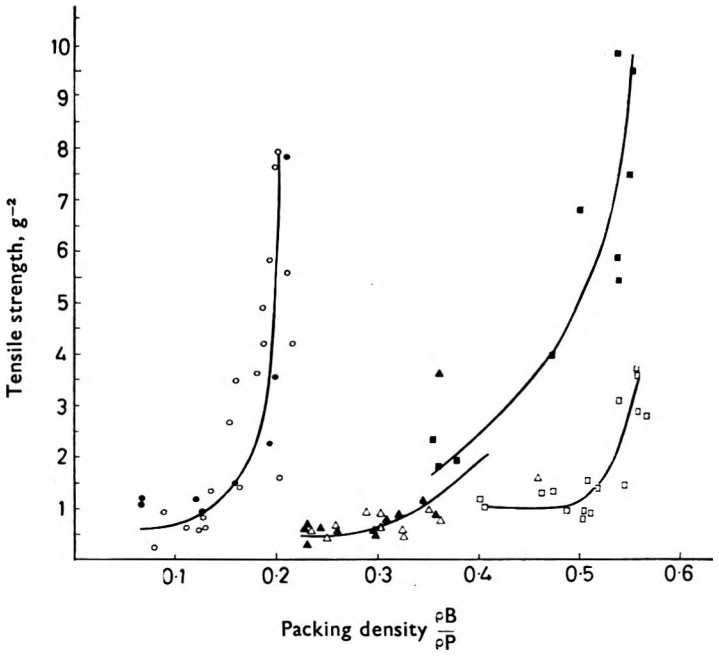

FIG. 8. Tensile strength of fine particle size materials. % w/w loss on drying calcium phosphate: ○, 1·01; ●, 1·8; lactose: △, 5·1; ▲, 5·27; potato starch: □, 17·7; ■, 23·2. RH (%) ○, △, □, 55; ●, ▲, ■, 86.

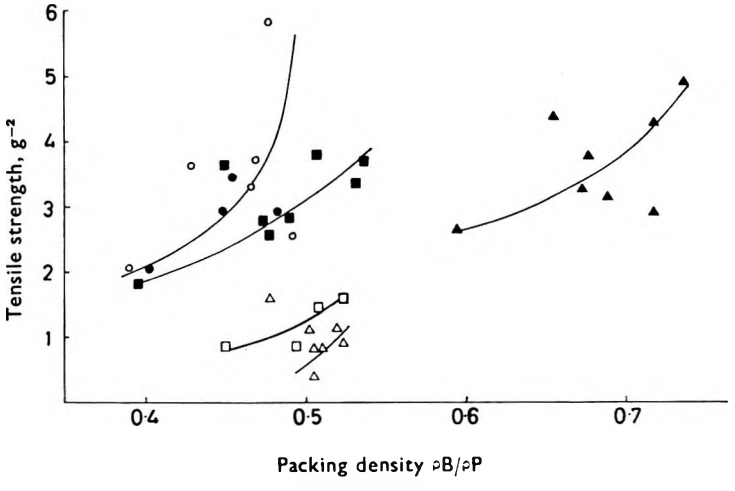

FIG. 9. Tensile strength of coarse particle size materials on exposure to high relative humidities. Potassium chloride; ○, 29 h; ●, 53 h at 88% RH. Sodium chloride: □, 4 h, ■, 52 h, at 76 → 80% RH. Sucrose △, 15 h; ▲, 98 h at 85% RH.

density, $\frac{\rho_B}{\rho_P}$ (Cheng, 1968), where ρ_B = bulk density g ml^{-1}, ρ_P = particle density g ml^{-1}.

From Fig. 8 it can be seen that for lactose and calcium phosphate, the small difference in moisture content at varying relative humidities does not produce a significant change in tensile strength. For potato starch, however, the higher equilibrium

moisture content at an elevated relative humidity causes an increase in tensile strength.

The results for sodium and potassium chloride and sucrose (Fig. 9) are more scattered but follow a similar pattern to those obtained for the other materials (Fig. 8). At low relative humidities, tensile strengths could not be measured on these materials due to their non-cohesive, free flowing nature. However, on exposure to high relative humidities, the values of tensile strength obtained were of equivalent magnitude to those for the more cohesive, fine particle size, bulk solids shown in Fig. 8.

DISCUSSION

It has been shown that free moisture exists in beds of bulk solids in at least two states; a 'pendular' state where liquid bridges occur between individual particles and a 'capillary' state where all the pores of the bed are filled with liquid which forms concave menisci at the pore ends. A 'transition' region between these two states is also suggested (Pietsch, 1967). For the thin beds of sodium chloride, potassium chloride and sucrose, the increase in moisture content, illustrated by Fig. 6, is accompanied by changes from the 'pendular' state through to the 'capillary' state. The significant changes in tensile strengths obtained for these materials, Fig. 9, may, therefore, be explained by the changes in the number and magnitude of the surface tension forces during the transition from various states of moisture location.

During moisture uptake the particle sizes of the sodium and potassium chloride and sucrose decrease by dissolution. The volume decrease of sodium chloride on the addition of 20% w/w water can be shown by calculation to be 7% (assuming that a saturated solution is formed). Thus, considering a median size of 214 μm, this would produce a median particle size of approximately 200 μm on dissolution. Since this size is still in the non-cohesive range the changes in tensile strength produced cannot be attributed solely to a reduction in size. The moisture must be evenly distributed throughout the material in the test cell for tensile strengths of beds determined in this manner to be meaningful. Table 5 shows the distribution of moisture at various depths in 1·1 and 6·2 cm-deep beds of sodium chloride. The results were obtained by exposing the upper surface only of the beds to a relative humidity of 90% for varying periods of time and determining the loss on drying of incremental sections. It can be seen that at low total moisture contents there is an uneven distribution of the water but as the moisture content increases reasonably uniform distributions occur, a higher moisture content being necessary in the thick beds for this state to be reached. This even distribution of moisture remains until the "capillary" state is reached when subsequent moisture uptake produces a supernatant layer.

Thus for those materials which do not exhibit a finite equilibrium moisture content, since their vapour pressures are below the partial pressure of the air–water mixture to which they are exposed, it is essential to use thin layers for humidification so that moisture distribution is uniform. For those materials that do reach an equilibrium moisture content at the relative humidities used, these bed depth considerations are less important, provided equilibrium is achieved. However, prolonged exposure times may be involved if the beds are excessively thick.

For potato starch the equilibrium moisture content obtained at 85% relative humidity agrees with the results obtained by Shotton & Harb (1965), but the tensile

Table 5. *Variation in moisture content throughout beds of sodium chloride* 1·1 *cm and* 6·2 *cm in depth*

Bed depths 1·1 cm			Bed depths 6·2 cm		
Total % w/w loss on drying	% depth from exposed surface	% w/w loss on drying between stated depth	Total % w/w loss on drying	% depth from exposed surface	% w/w loss on drying between stated depth
0·68	0		0·56	0	
		1·07			4·1
	25			5	
		0·99			1·9
	55			20	
		0·27			0·16
	100			41	
					0·06
3·49	0			61	
		3·9			0·11
	18			100	
		3·5			
	47		3·52	0	
		3·3			5·9
	71			12	
		3·2			5·3
	91			26	
		3·5			5·1
	100			46	
					4·5
				63	
					1·2
				77	
					0·5
				100	
8·75	0		5·30	0	
		8·9			5·8
	24			10	
		8·3			5·4
	49			26	
		8·3			5·3
	71			42	
		8·9			5·2
	94			59	
		11·6			5·1
	100			77	
					5·3
				100	
14·04	0				
		14·1			
	27		6·58	0	
		13·5			6·6
	50			13	
		13·9			6·1
	77			35	
		14·6			6·1
	96			49	
		15·7			6·3
	100			64	
					6·5
20·72	0			79	
		20·4			7·6
	25			100	
		20·1			
	47				
		20·8			
	72				
		21·4			
	96				
		21·9			
	100				

strengths obtained by Shotton & Harb (1966) at similar equilibrium moisture contents are lower than the values obtained in this work.

This is probably due to the use of a different packing density (not specified by Shotton & Harb, 1966) since their method of bed preparation involves dredging with no consolidation.

This emphasizes the importance of packing density as a variable in tensile strength determinations. A small change in the contact distance or co-ordination number of the particles in a bed of bulk solid will produce a significant change in the nature of interparticulate bonding. The shape of the curves in Fig. 8 illustrates this point and is confirmed by the results of Farley & Valentin (1967–68) and Aoki & Tsunakawa (1969).

The moisture present in the samples of lactose and calcium phosphate at equilibrium with high relative humidities is likely to approach the pendular state only; the tensile strengths are not significantly different.

Acknowledgement

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A stability study of chloramphenicol in topical formulations*

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The stabilities of several chloramphenicol preparations have been investigated at ambient temperatures. The B.P.C. ointment and eye ointment were stable over 2 years. The remainder retained more than 90% of their potencies after the following periods: ear drops B.P.C., 2 years, Drug Tariff cream, 5 months and eye drops B.P.C., 3 to 4 months. 15% hydrolysis occurred after autoclaving the eye drops, and 3 to 4% after heating with a bactericide. The latter sterilization process is recommended. Two assay procedures were employed, one estimating residual chloramphenicol by ultraviolet spectrophotometry after separation from decomposition products by thin layer chromatography and the other determining the main degradation product (1-*p*-nitrophenylpropan-1,3-diol-2-amine) colorimetrically.

The chloramphenicol molecule has several active functional groups but degradation in aqueous media is almost entirely due to hydrolysis of the amide group, giving rise to 1-*p*-nitrophenylpropan-1,3-diol-2-amine (the amine) and dichloroacetic acid (Higuchi, Marcus & Bias, 1954). The reaction follows first order kinetics and although the pH of maximum stability has been reported as about 6 (Trolle-Lassen, 1953; Broadhurst & Wright, 1959) it is largely independent of pH values between 2 and 7. Degradation rates rapidly increase in alkaline media and the reaction shows both specific and general acid-base catalysis, but is independent of ionic strength (Higuchi & others, 1954).

Although several fundamental studies have been reported on the nature of chloramphenicol degradation, little work has been published on the stability of the antibiotic in formulated preparations. Haemopoietic considerations limit the systemic use of chloramphenicol but it is still one of the most widely used antibiotics for local application. Several pharmacopoeias and formularies include details of topical dosage forms but frequently exclude an indication of shelf life. This paper describes stability studies on the topical formulations included in British reference compendia.

EXPERIMENTAL

Analytical methods

Amine method. This method was developed by Brunzell (1957) and involves the coupling of the amine produced on degradation of chloramphenicol with 1,2-naphthaquinone-4-sodium sulphonate and estimating the resulting colour photometrically.

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The residual antibiotic in degraded chloramphenicol mixtures can be calculated by subtracting the chloramphenicol molar equivalent of the amine from the original content.

In a preliminary experiment when 10 replicate samples of amine were assayed, the variation between the extreme figures was 2·7% and the maximum deviation from the mean 1·6%. This reproducibility was considered satisfactory, since its effect on figures for residual chloramphenicol is small, thus for example for a sample which is 10% degraded, the error in the residual chloramphenicol would be a tenth of that of the amine.

Experiments showed that neither the eye or ear drop vehicles nor the cream base (49% hard macrogol B.P.C. and 50% propylene glycol) interfered with the estimation.

Thin-layer chromatographic method. Since the amine method was not applicable to all samples, and under-estimated decomposition in heavily degraded samples, where secondary reactions become significant, a second method was used. This estimated residual chloramphenicol by spectrophotometry after a thin-layer chromatographic separation from decomposition products which also absorb in the ultraviolet region.

Schwarn, Dabner & others (1966) reported a thin-layer chromatographic separation of chloramphenicol from degradation products on Silica gel HF 254 using chloroform–isopropanol (80 : 20) as ascending phase. The spot was located under ultraviolet light at 254 nm, removed quantitatively and extracted with ethanol for spectrophotometric determination. Good agreement was reported between results from this assay and those obtained from two microbiological methods. Investigation of the technique showed that chloramphenicol, the amine, *p*-nitrobenzaldehyde and *p*-nitrobenzoic acid could be separated. The last two derivatives have been suggested, together with several condensation products, as intermediates in severely degraded chloramphenicol solutions (Knabe & Kraeuter, 1963; Lacharme & Netien, 1964).

Whenever possible, determinations using both analytical procedures were made in the shelf life studies and periodically results were compared with the cup plate assay.

Application of formulated preparations for thin-layer separation

The ophthalmic solution could be applied directly to the plate, as were the ear drops, after weight sampling and dilution. When the cream was spotted and run in the usual way a diffuse dumb-bell shaped spot formed near the origin and no partition occurred. Modification of the ascending phase failed to give adequate separation, so the cream was examined using Brunzell's method only. The ointment (1% in soft paraffin) and the eye ointment (1% in soft paraffin containing 10% wool fat) were dispersed in chloroform for application to the plate. The interference caused by the greasy bases was overcome by running the plate in two solvents. An initial run in ether took the vehicle to the top of the plate, while a second run in the usual solvent, until the front was just below the greasy layer, separated the chloramphenicol as before.

Quantitative removal of the spot was effected by scraping off the absorbent with a vulcanite spatula in a draught free environment into a funnel in a test-tube. Any adherent powder was first tapped then washed into the tube with ethanol. After 4 h, or overnight, when extraction was complete, the ethanolic solution was centrifuged for 5 min at 2000 rev/min and the supernatant estimated spectrophotometrically ($E_{1\%}$, 1 cm chloramphenicol in ethanol at 274 nm = 308). Schwarn's description of the process (1966) contains few practical details. Those we used together with the reproducibility of the results, are given in Table 1.

Table 1. *Thin-layer chromatographic assay of chloramphenicol preparations with recoveries and practical details*

Preparation	Pre-treatment of sample	Volume spotted (μl)	Volume of ethanol for extraction (ml)	Recovery %	Coefficient of variation: Single assay	Coefficient of variation: Mean of 4 assays
Eye drops (0·5%)	None	25	10	97·1	3·3	1·7
Ear drops (5%)	1 g in water adjusted to 20 ml	25	5	97·1	3·3	1·7
Eye ointment (1%)	0·70 g in chloroform adjusted to 10 ml	50	5	97·5	8·1	4·1
Ointment	0·70 g in chloroform adjusted to 10 ml	50	5	97·4	9·5	4·8

During the stability investigations all assays were made in quadruplicate. Each time a sample was submitted for assay, fresh standard solutions of chloramphenicol were also run in quadruplicate and the percentage recovery calculated. The mean recovery of standards during the investigation was 97·6% and the extreme values were 95·3 and 101%. The recoveries of the unknown were assumed to equal those of the standards.

Stability investigation

Chloramphenicol ear drops B.P.C. A sample was prepared, assayed by direct spectrophotometry, packed in a dry actinic stoppered bottle and stored at 20–25° for about 2 years. Samples were withdrawn at intervals and assayed using the procedures described above. Results are in Table 2.

Table 2. *The stability of some chloramphenicol topical formulations*

	Chloramphenicol remaining %							
	Ear drops		Cream (Drug Tariff) formula	Eye drops: Refrigeration 0–4°		Eye drops: Room temperature 20–25°		
Storage time (weeks)	Amine method (99·7)*	TLC method	Amine method (110)*	Amine method (99·6)*	TLC method	Amine method	(99·6)*	TLC method
0	100	99·6	100	100	100	100		100
0	—	—	—	97·2†	98·1†	97·2†		98·1†
4	—	—	98·6	—	—	—		—
8	98·5	96·7	96·6	96·9	95·6	94·3		94·3
16	98·0	94·7	94·2	96·6	98·1	91·5		90·5
20	—	—	93·0	—	—	—		—
24	97·7	96·1	91·2	96·5	93·5	83·3	(92·4)‡	88·3
32	—	—	90·2	—	—	—		—
40	96·9	95·7	87·5	95·9	95·0	81·6		81·1
48	—	—	85·0	—	—	—		—
52	95·9	97·4	84·0	96·1	94·4	77·9	(77·8)‡	75·7
76	94·5	94·5	—	95·0	93·2	71·6		67·2
88	94·9	94·6	—	94·5	94·9	69·0		67·0
96	96·5	93·7	—	94·6	97·3	64·0		68·9

*Initial assay by direct spectrophotometry.
†After sterilization.
‡Cup plate assay.

Chloramphenicol cream. Drug Tariff Formula. After its preparation and assay, the sample was stored in a screw capped actinic glass jar at 20–25° C. The results of assays at various intervals are in Table 2. The marked decrease in stability of the cream compared with the ear drops is surprising, since the only difference in formulae is the replacement of 49% of propylene glycol by hard macrogol B.P.C., to solidify the preparation. It was established, by subjecting carefully dried raw materials to suitable challenges, followed by factorial analysis, that the increased degradation was due to moisture, and that the macrogol had no effect on the reaction. A sample of hard macrogol B.P.C. used in the cream gave a figure of 1·2% water compared with 0·21% for the propylene glycol used in the eardrops. These results together with the known hydrophilic nature of the macrogols support the conclusions.

Chloramphenicol eye ointment and ointment B.P.C. Samples of the eye ointment, prepared aseptically, and the ointment were assayed both by the B.P.C. extraction method and the TLC method described previously. The ointments were packed in screw capped actinic glass jars, from which aliquots were withdrawn at intervals for TLC analysis. The results obtained gave no evidence of degradation within the limits of reproducibility of the assay. Confirmation of this was given by qualitative examination of the thin-layer plates, which showed no new spots over the two year study period.

Chloramphenicol eye drops B.P.C. A preliminary study was made using Brunzell's method to assess the possibility of heat sterilization of these drops, which are currently processed by filtration. Sterilization by heating with a bactericide (the phenylmercuric nitrate present in the formulation) at 100° for 30 min resulted in 3–4% hydrolysis, while autoclaving at 115° for the same period resulted in about 15% degradation. In neither case was any degradation product other than the amine seen on the thin-layer chromatogram. Heat treatment was followed by rapid cooling in both cases. The results of a stability investigation at two temperatures on a sample of eye drops packed in ampoules and sterilized by heating with a bactericide are shown in Table 2.

DISCUSSION

Shelf lives at 20 to 25°, can be inferred from the above results. The ointment and the eye ointment can be expected to retain their potencies for over two years. The ear drops and the cream, provided precautions are taken to exclude moisture can be given anticipated shelf lives of two years with less than 10% overage. However, since it is difficult to completely exclude moisture from the cream, 5 months is probably a more realistic estimate. A shelf life of two years can be predicted for the eye drops with 10% overage if stored at 0 to 4°, but only 3 to 4 months can be recommended at 20 to 25°.

The eye drops study may rationalize the very different approaches adopted for chloramphenicol ophthalmic solutions in various pharmacopoeias and formularies. For example, the United States Pharmacopeia 17 prescribes reconstitution of freeze dried buffered powder, the British Pharmaceutical Codex 1968 and the Australian Pharmaceutical Formulary 1964 recommend sterilization by filtration, while the Pharmacopoeia Nordica 1963 sterilizes the drops at 100° for 20 min. The results confirm the suitability of heat sterilization with a bactericide at 100° followed by rapid cooling. Since such solutions retain potency for considerable periods, particularly on

refrigeration, perhaps the British Pharmaceutical Codex should give consideration to this alternative method of manufacture.

The estimates of shelf life data presented above would only be valid if the amine formed on decomposition were non-toxic. The possibility of local or systemic toxicity seems little more than a theoretical hazard since the amine is a normal metabolite of chloramphenicol (Glazko, Dill & Rebstock, 1950). Further, at the Birmingham and Midland Eye Hospital, all chloramphenicol eye drops have been autoclaved for some 3 years, and are still considered to be one of the blandest eye preparations available (Roper-Hall: personal communication). Similarly, intrathecal chloramphenicol, although rarely used these days is frequently sterilized by autoclaving, apparently without the development of adverse reactions.

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A borax-chloramphenicol complex in aqueous solution

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A 1,2-complex between borax and chloramphenicol occurs in aqueous solution. The relative stabilities of simple aqueous solutions of chloramphenicol and the B.P.C. eye-drops are explained by the existence of this complex.

The kinetics of the hydrolysis of chloramphenicol in aqueous solution have been investigated by Higuchi, Marcus & Bias (1954) who showed that the reaction was general acid-base catalysed, and was slowest and substantially independent of pH between 2 and 7. It is therefore necessary to buffer aqueous chloramphenicol solutions within this range, and borax and boric acid are used for this purpose in the B.P.C. eye-drops. These substances appear to have a more specific effect on chloramphenicol than most buffers, thus Fenton (1955) showed that chloramphenicol is more soluble in 0·6% borax solution than in either water or 0·6% sodium carbonate, and Broadhurst & Wright (1959) found that chloramphenicol was nearly 5 times more stable in borax buffer than in phosphate buffer, even though both had the same pH.

Boric acid is known to complex with hydroxy compounds (Sciarra & Elliott, 1960). An investigation was therefore undertaken to determine if the influence of the borax-boric acid buffer of the B.P.C. eyedrops on chloramphenicol could be due to the existence of a similar complex.

EXPERIMENTAL

Thin-layer chromatography. The procedure has been described (James & Leach 1970).

Solubility determinations. Excess chloramphenicol was shaken at the required temperature with solutions of borax or boric acid of the required strengths until saturated. Preliminary experiments showed that 4 h were sufficient. Samples of saturated solution were withdrawn and assayed spectrophotometrically at 274 nm. The degree of hydrolysis during the 4 h equilibrium period was less than 1% and was therefore disregarded.

pH determinations. pH values of solutions of chloramphenicol with boric acid and of chloramphenicol with borax were measured using a Pye-Unicam 290 pH meter. Concentrations of borax and boric acid were kept constant throughout, and precautions were taken to exclude carbon dioxide.

Determination of decomposition rates. Samples of a 0·05% solution of chloramphenicol in water were packed in 5 ml ampoules and immersed in a constant temperature bath for 6 h. Samples were withdrawn at hourly intervals, and assayed by the amine method, described previously (James & Leach, 1970).

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DISCUSSION

Thin layer chromatography of fresh aqueous solutions of chloramphenicol gave one spot, but freshly prepared solutions in the borax-boric acid buffer gave two, the upper spot corresponding to that of the aqueous solution, indicating that chloramphenicol exists in the borax-boric acid buffer in a form not encountered in simple aqueous solutions.

pH determinations revealed no evidence for complexation of chloramphenicol with either borax or boric acid. Solubilities were not influenced by the presence of boric acid, but increased with borax concentration. These results were not directly due to the effect of borax on pH since there is no correlation between solubilities and pH values (Table 1). Confirmation was obtained by determining solubilities in a range of glycine buffers between pH 6·9 and 9·1 and observing that the solubility was constant.

Table 1. *Solubility of chloramphenicol in borax solutions*

Molar borax concentration	Solubility %	pH of solution
0	0·375	4·70
0·0001	0·391	7·15
0·001	0·438	8·65
0·005	0·614	8·65
0·01	0·732	8·65
0·02	1·23	8·65
0·05	2·14	8·70
0·11	3·46	8·90
0·125	3·67	8·90
0·15	3·87	9·00

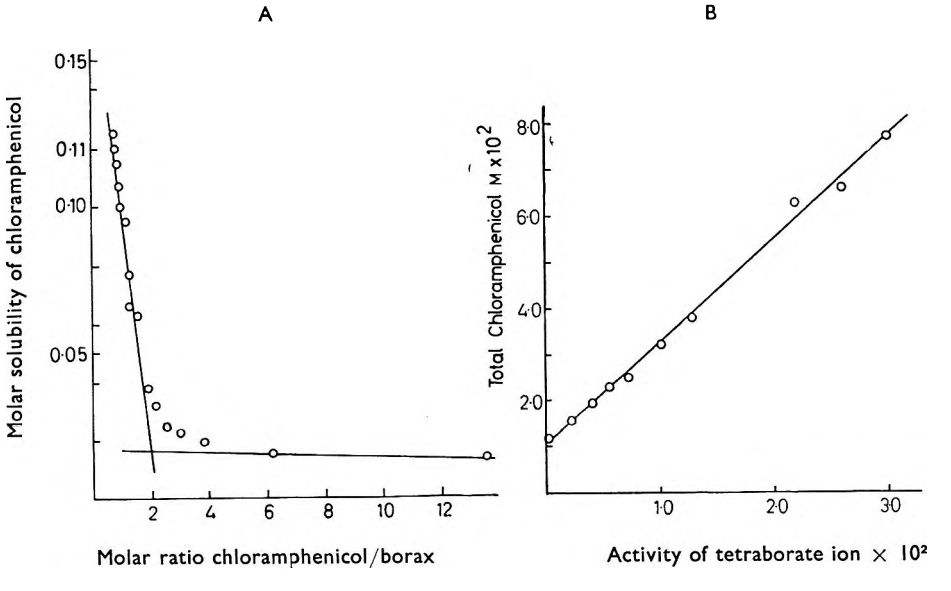

FIG. 1A. Determination of stoichiometric ratio of chloramphenicol to borax.
B. Solubility of chloramphenicol in aqueous borax solutions at 25°.

Solubilities at 25·5° are plotted against the molar ratio, chloramphenicol/borax, in Fig. 1A. The graph shows an inflection at a ratio of 2, suggesting the equilibrium,

$$2C + B \overset{K_1}{\rightleftharpoons} C_2B \tag{1}$$

where C represents chloramphenicol, and B borax.

A plot of total chloramphenicol in solution (C_t) against total borax (B_t) is linear for this type of equilibrium, with an intercept (C_o), representing the solubility of chloramphenicol in water. The actual graph was curved and presumed to be a consequence of non-ideal behaviour of borax. The mean activity coefficients (f) of electrolyte solutions of ionic strength (μ) up to about 0·25, are given by Debye-Hückel theory as,

$$\log f = \frac{Az^+ z^- \sqrt{\mu}}{1 + \sqrt{\mu}} \tag{2}$$

where z represents the charges on the respective ions, and A is a temperature dependent constant, having a value of 0·51 at 25°. The graph of total chloramphenicol against tetraborate ion activity, calculated from equation (2) is in fact linear, as shown in Fig. 1B, confirming equilibrium (1). A similar plot was obtained with the 37° results.

The quantity of uncomplexed chloramphenicol (C_o) is limited by its aqueous solubility, and is constant, so that the equilibrium can be expressed as,

$$K = \frac{[C_2B]}{C_o^2\,[B]} \tag{3}$$

$[C_2B]$ can be placed equal to $\frac{1}{2}(C_t - C_o)$, and uncomplexed borax concentration [B] calculated as $[B_t - \frac{1}{2}(C_t - C_o)]f$. Substitution in equation (3) gave mean equilibrium constants of $2{\cdot}9 \times 10^4$ at 25° and $1{\cdot}1 \times 10^4$ at 37°. An approximate heat of complexation of 67 kJ mol^{-1} was obtained from these results. If this is assumed to be independent of temperature, an equilibrium constant of about 100 would be anticipated at 100°. Chloramphenicol solutions for intrathecal injection or for irrigation of infected wounds are sometimes sterilized by autoclaving. Such solutions are not usually buffered, and were therefore expected to decompose more rapidly than the eye-drops. In contrast, rate determinations gave first order constants of $7{\cdot}74 \times 10^{-4}$ min^{-1} at 100° and $2{\cdot}01 \times 10^{-3}$ min^{-1} at 115°, compared with $1{\cdot}15 \times 10^{-3}$ and $3{\cdot}03 \times 10^{-3}$ respectively, for the eye-drops. Any stabilizing effect due to complexation will be reduced by general acid-base catalysis by the buffer ions. The poor stability of the eye-drops at elevated temperatures, compared with that of the simple aqueous solution, probably arises because the degree of complexation is so small in this region that the second effect predominates.

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LETTERS TO THE EDITOR

The polymorphism of aspirin

Mitchell & Saville (1967) demonstrated that various samples of commercial aspirin have different rates of dissolution. Subsequently, Tawashi (1968, 1969) has reported the existence of aspirin in two polymorphic forms (designated Forms I and II). During the course of present work concerned with the tabletting characteristics of various polymorphic forms of drugs, we have been able to identify several aspirin polymorphs in addition to those already reported. Table 1 shows the conditions under which the polymorphs were obtained.

Confirmatory evidence that all the samples examined were aspirin and differed only in crystalline form is afforded by the fact that no differences could be detected in their ultraviolet spectra when dissolved in ethanol (determined using a Unicam SP800 recording spectrophotometer).

A Perkin-Elmer Differential Scanning Calorimeter DSC-1 equipped with an effluent analyser was used for the thermal analysis of the polymorphs. Samples weighing between 2 and 10 mg were analysed using a scanning rate of 8° min^{-1}. No traces of solvent of crystallization were detected by the effluent analyser when any of the aspirin samples were fused. This therefore excludes the possibility that the samples were solvated forms of aspirin. The DSC traces for the six polymorphs are shown in Fig. 1.

A Kofler hot stage microscope was used to confirm the melting points of the polymorphs. The results are shown in Table 1. The instrument was also used to observe solution phase transformations of pairs of polymorphs. The solvent for this work was n-pentanol saturated with one component of the particular polymorph pair under study. Some of the phase transformations are summarized in Table 2.

Since different polymorphs have different crystal packing conditions, it is to be expected that they will have different true densities. Density determinations were made at 20° using a specific gravity bottle with a displacement medium of light liquid paraffin. Table 1 contains the results of this work.

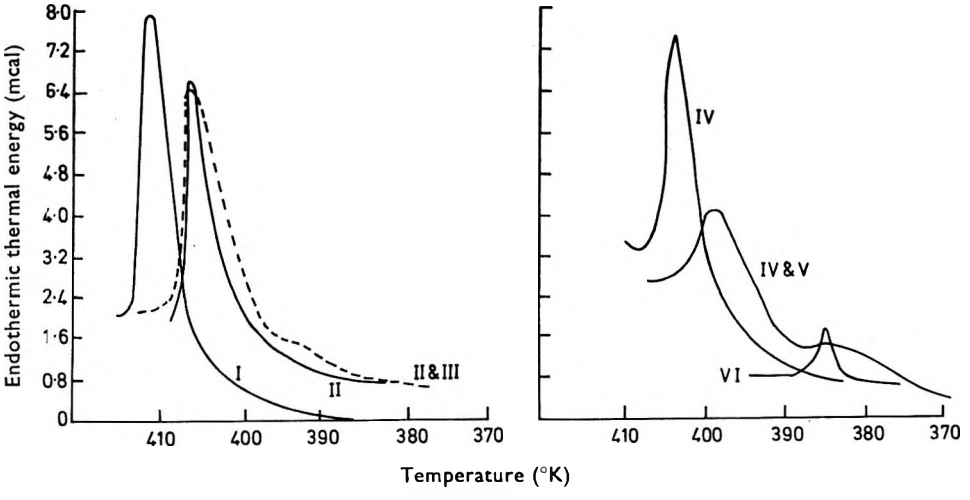

FIG. 1. DSC traces of the six polymorphs of aspirin.

Table 1. *Methods of preparation and physical characteristics of aspirin polymorphs*

Method of preparation	Form nomenclature	Melting point (°C) DSC	Melting point (°C) Hot stage microscope	Density at 20° C (g ml^{-1})
Slow recrystallization from 96% ethanol at 20°	Form I	135	133	1·40
Slow recrystallization from n-hexane at 0°	Form II	129	128	1·50
Slow recrystallization from n-hexane at 20°	Form II & Form III	123 & 114	124 & 115	*
Slow recrystallization from n-octane at 20°	Form IV	123	121	1·36
Slow recrystallization from n-octane at 0°	Form IV & Form V	119 & 100	118 & 100	*
Sublimation of aspirin vapour under vacuum	Form VI	108	110	1·29

* Not possible to isolate polymorphs and make density determinations.
The melting points of polymorphs prepared by Tawashi (1968) were Form I 143° and Form II 125°.

Table 2. *Solution phase transformations of polymorph pairs at 20° C in n-pentanol*

Pair of polymorphs examined	Transformation observed
Form I + Form II	Form II ⟶ Form I
Form II + Form III	No transformation
Form I + Form IV	Form IV ⟶ Form I
Form II + Form IV	Form II ⟶ Form IV

X-ray powder diffraction (Phillips PW 1009/30) using nickel-filtered copper radiation was also performed on the polymorphs. However, only minor differences in the number and intensity of the lines in the diffraction patterns were observed.

The aspirin polymorphs were stable in the presence of moisture and upon storage. Grinding of the polymorphs in a Glen Creston micro-ball mill had no effect with the exception of Form III that was transformed to Form II.

Further work is continuing on the characterization of the polymorphs using dissolution techniques.

The authors would like to thank Dr. J. D. Donaldson for helpful discussions concerning the X-ray powder diffraction studies.

One of us (M.P.S.) would like to thank the Science Research Council for the award of a Research Studentship.

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Treatment of adjuvant arthritis with antibradykinin drugs

The discovery of the polypeptides of the bradykinin type has made possible an additional approach to the problem of mediation of inflammatory reaction. Besides such mediators as histamine and 5-hydroxytryptamine, bradykinin could be responsible for some syndromes of inflammation particularly for the pathogenesis of the delay phase of the reactions (Turk & Willoughby, 1969).

We now present the results of experiments on bradykininogen and the influence of some antibradykinin drugs on the course of rheumatic-like pathology in rats. Both the induction procedure and the gradation of its course were described by Pelczarska (1969).

To estimate the level of bradykininogen in blood plasma in adjuvant-treated rats, seven groups of eight randomly bred Wistar rats were used. The animals were injected with complete Freund adjuvant and at appropriate intervals each group of animals was bled. Bradykininogen levels were estimated according to Diniz, Carvalho & others (1961).

The following pattern of examination was used. Group I: animals in the induction phase of immunological response (24 h after injection of adjuvant); Group II: animals in the latent period of the condition (7th day); Groups III–V: rats in the period of full manifestation of the condition (17th, 22nd, and 30th day, respectively); Group VI: after recovery (40th day); Control group (non-arthritic rats). The results for bradykininogen are shown in Fig. 1A.

To test the therapeutic effects of anti-inflammatory drugs, possessing antibradykinin activity, adjuvant-treated rats of randomly bred Wistar strain were treated with indomethacin (1 mg/kg, orally), sodium phenylbutazone (8 mg/kg, i.p.), amidopyrine (3 mg/kg, i.p.) and sodium salicylate (65 mg/kg, orally). The drugs were administered one day before Freund adjuvant and then three times weekly. The results are in Fig. 1B.

Antibradykinin activity of the drugs was tested both *in vivo* and *in vitro*.

The *in vivo* experiments were made on inbred August rats. The animals had been pretreated as described above with daily doses of the drugs for 3 days before experiments started (on the 3rd day, 1 h before testing). The pretreated and control

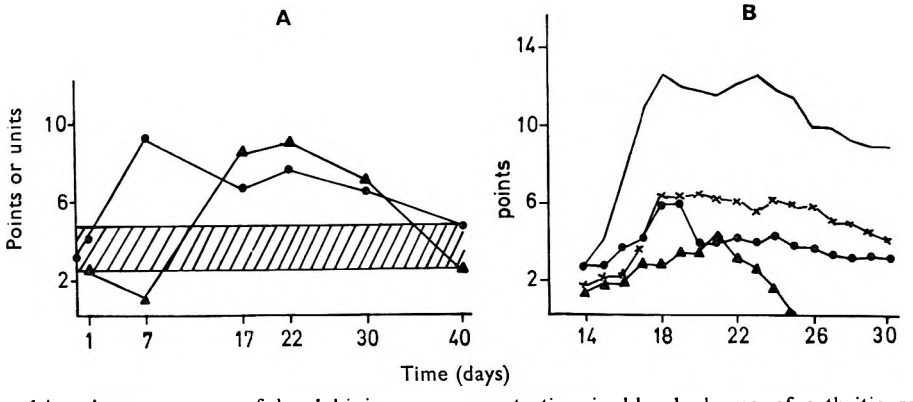

FIG. 1A. Average mean of bradykininogen concentration in blood plasma of arthritic rats (—▲—) in units of bradykinin (ordinate) formed per ml of plasma. Intensity of arthritic manifestation (—●—) as scored according to a conventional 18 point pattern (ordinate) (average mean in points see Giełdanowski, Pelczarska & others, 1969). Hatched area is control level of bradykininogen.

B. Influence of drugs on the course of adjuvant arthritis. —●— Indomethacin; —×— Phenylbutazone; —▲— Amidopyrine; —— Control. Ordinate: points as in Fig. 1A.

Table 1. *Influence of drugs on skin reaction to bradykinin and their antibradykinin activity* in vitro. Skin reaction to bradykinin was assessed on the abdomen surface of prepared skin flaps as follows: the area of brown coloured patch (in cm^2) was multiplied by the number of points (1–4 according to the intensity of the skin reaction). Average means. The concentration of the drugs in the *in vitro* test are relative to the equivalent of LD50 of indomethacin, the therapeutic concentration of which was assumed to be 10 μg/ml

		In vitro test	
Drugs	Skin reaction (inhibition %)	Concentration of drugs (μg/ml)	Inhibition (%)
Indomethacin	68	10	0
Phenylbutazone ..	67	3	0
Amidopyrine	93	3·3	30
Sodium salicylate ..	59	0·05	12

rats were then injected intradermally in the skin of the abdomen with 0·1 ml of bradykinin (100 μg/ml) and in the other flank with 0·1 ml of physiological solution of saline. Simultaneously the animals received intravenously 1% colloidal silver (1 ml/100 g) for the visualization of the inflammatory region in the skin (Jancsó, 1961).

The animals were killed 3 h later and the skin reaction to bradykinin was scored (Table 1).

Bradykininogen concentrations in blood plasma increased markedly, particularly in the latent period of the disease (Fig. 1). The means of bradykininogen values during the period of full manifestation of the inflammatory condition were about twice as high as normal and then (on the 40th day) returned to the threshold value.

Houssay, Monfort & others (1964) had found an increase of globulin and diminution of the albumin fraction of plasma proteins as the condition developed. Again, Lowe (1964) reported the appearance of a factor migrating (in immune-electrophoresis) near α_2-globulin fraction at the period of between 16–20th day after the injection of adjuvant. So the question arises whether the augmentation of quantity of the bradykinin precursor in plasma is really significant in the pathogenesis of the adjuvant induced condition.

All the drugs tested showed significant antibradykinin activity in the skin reaction test, and amidopyrine and to some degree sodium salicylate in the *in vitro* test.

But among the drugs, indomethacin, amidopyrine (in doses of 1/75 of the LD50) and phenylbutazone (in doses of 1/25 LD50), showed favourable effects upon the arthritis syndrome in the adjuvant-treated rats. Sodium salicylate did not influence the course of the condition.

The results of the experiments indicate that bradykinin can play a role in the inflammatory process, but antibradykinin properties of drugs need not be correlated with their anti-arthritic activity, as was shown with sodium salicylate.

The increase of bradykininogen level in blood plasma seems to have a significance in the pathology of adjuvant arthritis influenced by intracutaneous injection of complete Freund adjuvant in rats.

Our thanks are due to Miss J. Lewandowska for her skilful technical assistance.

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Inhibitory effect of *p*-hydroxyphenylisopropylarterenol on the isolated human myometrium

There is a need for drugs to suppress excessive uterine activity of premature labour. Compounds with β-adrenergic receptor stimulating properties have been synthesized and tested for this action *in vitro* and *in vivo*. One of the most active seems to be *p*-hydroxyphenylisopropylarterenol (Cc 25; Philips-Duphar). In this paper two characteristics not previously described are presented*.

Myometrial strips from patients undergoing hysterectomy, legal abortion or Caesarean section, were mounted in an isolated organ bath and the motility recorded on a smoked drum (Bygdeman & Eliasson, 1963). The drug was dissolved in normal saline and fresh solutions were prepared immediately before use because at neutral pH there was a rapid auto-oxidation.

Myometrial strips ($n = 50$) at late proliferatory phase from 16 non-pregnant patients showed a clear inhibition (50% or more) of the amplitude or frequency of the contractions, or both, with the drug at $1–2{\cdot}5 \times 10^{-7}$ g/ml. When the spontaneous activity had been restored after washing, the myometrium was always completely refractory to a second dose, even if this was 10 times larger than the first (Fig. 1). A subsequent dose of PGE_1 always inhibited the motility indicating a normal reactivity to other inhibitors.

Myometrial strips ($n = 16$) from four patients in the 12th to 20th week of gestation responded qualitatively in the same way as those from the non-pregnant patients but the sensitivity was 100–1000 times higher, i.e. a clear inhibition could be obtained with $0{\cdot}1–1 \times 10^{-9}$ g/ml. The tachyphylaxis was not as complete as for the non-pregnant myometrium (Fig. 2).

Myometrial strips from patients at term were less sensitive to the drug than those from non-pregnant women. In one experiment (three strips from one patient) a clear inhibition was obtained with $0{\cdot}1 \times 10^{-6}$ g/ml, while in two experiments (five strips) no effect was noted with $0{\cdot}25–0{\cdot}75 \times 10^{-6}$ g/ml. Doses up to 1×10^{-5} g/ml were tested, but were always without effect. Whether this arose from primary insensitivity or tachyphylaxis could not be ascertained.

Propranolol (10^{-5} g/ml) completely blocked the effect of the drug.

The effects of the drug on the human uterus *in vitro* have also been described by

* Presented at an International Symposium on Uterine Physiology and Pharmacology, June 20–22, 1968, New York.

FIG. 1. Inhibitory effect of the drug (0·3 μg/ml) on the spontaneous motility of the isolated human non-pregnant myometrium. Note the lack of effect of the second dose.

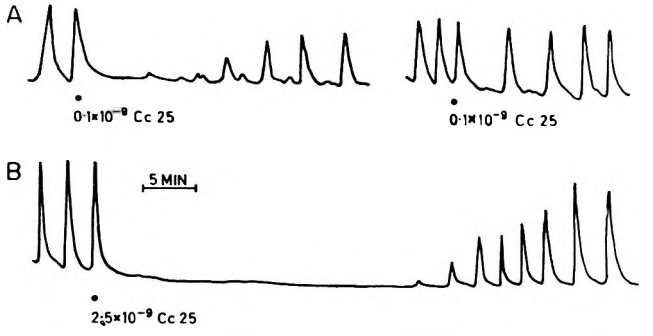

FIG. 2. Inhibitory effect of the drug (0·1 and 2·5 ng/ml) on two strips of myometrium from a woman in the third month of pregnancy. The experimental conditions as in Fig. 1.

Nakanishi, McLean & others (1969) but my results are at some variance with theirs, since they noted that the pregnant myometrium at term was more sensitive than the non-pregnant myometrium. The sensitivity of the non-pregnant uterus was the same in both studies.

Despite the marked tachyphylaxis *in vitro* there seems to be no escape phenomenon when the drug was given intravenously to suppress the uterine activity (Stolte, Eskes & others, 1965; Wansbrough, Nakanishi & Wood, 1968).

The reason for the significant increase in sensitivity during the early stage of pregnancy is not clear, but it could be related to the change in adrenergic innervation of the uterus that takes place during pregnancy (Sjöberg, 1967).

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Effect of some arrhythmogenic agents upon the acetylcholine content of the rabbit atria

There is evidence for the importance of acetylcholine as an essential cellular constituent of cardiac tissue affecting cationic exchange and rhythmicity (Burn, 1969), and recently the acetylcholine content of the heart after drug-induced changes has been measured (Malhotra & Pundlick, 1965; Sharma & Parmar, 1967). We now report the effect of three arrhythmogenic agents, aconitine, digitalis and barium, on the amount of acetylcholine in the isolated atria of the rabbit.

Thirty-eight albino rabbits of either sex, 1·3–2·4 kg, were stunned by a blow on the head, and the hearts removed. Atria were dissected from surrounding tissues and suspended at 29° ± 0·5° in a 40 ml bath containing oxygen saturated Ringer-Locke solution containing (g/litre): NaCl 9·0, KCl 0·42, $CaCl_2$ 0·24, $NaHCO_3$ 0·5 and dextrose 1. Contractions were recorded kymographically by a spring lever with minimal inertia. After the preparation was allowed to equilibrate for 60 min, 0·2 to 0·4 ml of freshly prepared solution of the arrhythmogenic agent in Ringer-Locke was added to make a final concentration in the bath of aconitine nitrate, 10 μg/ml, deslanoside 10 μg/ml, barium chloride 200 μg/ml. This resulted in the development of arrhythmia as seen kymographically, and, in a few instances confirmed electrocardiographically. When the arrhythmia had lasted for 5 min, atria were removed for estimation of their acetylcholine content (Anand, 1952). The extraction of acetylcholine was made in 10 ml of eserinized Ringer solution at pH 4 at 90° to 100° (Anand, 1952). The assay was with the frog rectus abdominis muscle. All the drugs caused a statistically significant increase in the acetylcholine content of the atria (Table 1).

Table 1. *Acetylcholine content (μg/g of fresh tissue) of the rabbit atria during drug-induced arrhythmia*

Drug	Dose μg/ml	Number of rabbits	Mean ± s.e.	Significance value
Control	—	12	1·29 ± 0·23	—
Aconitine nitrate	10	8	1·96 ± 0·18	$P < 0·05$
Deslanoside	10	8	8·66 ± 1·08	$P < 0·001$
Barium chloride	200	10	6·34 ± 1·05	$P < 0·001$

Acetylcholine causes a loss of potassium from the heart resulting in electrophysiological changes conducive to the development of arrhythmias; quinidine depresses this cationic efflux. On this basis Holland (1957) believes quinidine to act by interfering with the acetylcholine system in the heart. Other antiarrhythmic drugs like propranolol, diphenylhydantoin and pentobarbitone share with quinidine the ability to reduce the acetylcholine content of the heart (Khanna & Madan, 1968; Madan & Khanna, 1970). Also, diphenylhydantoin converts a digitalis-induced arrhythmia to sinus rhythm with a corresponding reversal of the digitalis-induced potassium efflux (Scherlag, Helfant & others, 1968). Our experiments are consistent with the view that acetylcholine may also be involved in the production of cardiac arrhythmias by drugs.

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Effect of apomorphine and pimozide on synthesis and turnover of labelled catecholamines in mouse brain

Labelled tyrosine has been used for the study of catecholamine synthesis and turnover in brain *in vivo* (Udenfriend & Zaltzman-Nirenberg, 1963; Gordon, Reid & others, 1966; Sedvall, Weise & Kopin, 1968; Nybäck & Sedvall, 1970). This method has the advantage that concentrations of endogenous amines in brain are left unchanged. We have now examined the effects of apomorphine and pimozide on accumulation and disappearance of catecholamines formed in mouse brain from [^{14}C]tyrosine.

Apomorphine stimulates dopamine receptors in rat brain (Ernst & Smelik, 1966; Ernst, 1967). Andén, Rubenson & others (1967) and Roos (1969) presented evidence that apomorphine decelerates dopamine turnover in rat brain, possibly by activating a negative feed-back mechanism from the stimulated receptors. In a recent study Persson & Waldeck (1970) obtained results indicating that apomorphine accelerates noradrenaline turnover in mouse brain.

Pimozide is a potent neuroleptic drug (Sterkmans, Brugmans & Gevers, 1968; Haase, Blankenburg-Zahn & others, 1969) and is more effective than chlorpromazine and haloperidol in antagonizing apomorphine-induced stereotyped behaviour (Janssen, Niemegeers & others, 1968). This indicates that the drug is a dopamine receptor blocker. Chlorpromazine and haloperidol accelerate synthesis and turnover of catecholamines in brain (Carlsson & Lindqvist, 1963; Corrodi, Fuxe & Hökfelt, 1967; Nybäck, Borzecki & Sedvall, 1968), effects which probably are due to an activation of the presynaptic neuron as a consequence of the receptor blockade.

After an intravenous injection of [^{14}C]tyrosine to mice, the contents in brain of labelled dopamine and noradrenaline increase during the first 30 min (Nybäck & others, 1968). Between 2 and 7 h after the precursor administration, the labelled amines disappear from brain at rates that appear to be exponential and are not altered by synthesis inhibition with α-methyltyrosine (Nybäck & Sedvall, 1970). Thus, the disappearance of labelled amines during the mentioned time interval will be determined predominantly by the turnover rates of the amines.

Saline or drugs were administered 2 h after the intravenous injection of [^{14}C]tyrosine (10 μCi/animal, 355 mCi/mmol). Groups of animals were killed 2 and 7 h after the precursor administration and the contents in brain of endogenous tyrosine and labelled tyrosine, dopamine and noradrenaline were measured (Nybäck & Sedvall, 1970). In a separate experiment the effect of apomorphine and pimozide on endogenous dopamine and noradrenaline concentrations in brain was measured spectrophotofluorimetrically (Bertler, Carlsson & Rosengren, 1958; Carlsson & Waldeck, 1958).

Apomorphine reduced the content of endogenous noradrenaline to about 70% of controls, whereas pimozide caused a reduction of the dopamine level in mouse brain (Table 1).

Apomorphine retarded whereas pimozide accelerated the rate of disappearance of [^{14}C]dopamine from brain in comparison with saline-treated animals (Table 2).

None of the drugs significantly changed the rate of disappearance of [^{14}C]noradrenaline or the specific activity of [^{14}C]tyrosine.

When [^{14}C]tyrosine was administered by constant rate intravenous infusion for 20 min, the accumulation of labelled dopamine was decreased by apomorphine but increased by pimozide (Table 3). None of the drugs significantly altered the accumulation of [^{14}C]noradrenaline or the specific activity of [^{14}C]tyrosine.

Our results show that turnover of brain dopamine can be accelerated or decelerated by treatment with drugs. The evidence in Table 2 shows that the rate constant for dopamine turnover in brain can be altered more than twice.

Table 1. *Levels of tyrosine and catecholamines in mouse brain 1 h after treatment with saline, apomorphine* (25 mg/kg, i.p.) *or pimozide* (2 mg/kg, orally). Figures represent mean values for groups of five animals ± s.e.

Treatment	Tyrosine μg/g	Dopamine μg/g	Noradrenaline μg/g
Saline	17 ± 1·4	0·59 ± 0·028	0·38 ± 0·014
Apomorphine	16 ± 1·2	0·62 ± 0·039	0·27 ± 0·013†
Pimozide..	18 ± 2·9	0·50 ± 0·026*	0·41 ± 0·027

* Differs from saline group ($P < 0·05$).
† Differs from saline group ($P < 0·001$).

Table 2. *Effect of apomorphine* (25 mg/kg, i.p.) and *pimozide* (2 mg/kg, orally) *on disappearance of catecholamines formed from* [^{14}C]*tyrosine in mouse brain.* Saline or drugs were administered 2 h after the i.v. injection of [^{14}C] tyrosine Animals were killed 2 and 7 h after [^{14}C]tyrosine administration. Rate constants (k) for the disappearance of labelled amines were calculated according tc the method of least squares. Figures represent the mean values for groups of 5–7 animals ± s.e.

Treatment	Time h	[^{14}C] Dopamine counts/min g^{-1}	^{k}[^{14}C]-dopamine	[^{14}C] Noradrenaline counts/min g^{-1}	^{k}[^{14}C] Noradrenaline
—	2	2020 ± 160	—	443 ± 46	—
Saline	7	691 ± 39	0·21 ± 0·020	174 ± 11	0·18 ± 0·025
Apomorphine	7	978 ± 38*	0·14 ± 0·019†	157 ± 13	0·20 ± 0·027
—	2	1590 ± 93	—	437 ± 42	—
Saline	7	552 ± 19	0·21 ± 0·013	176 ± 13	0·18 ± 0·024
Pimozide	7	266 ± 30*	0·36 ± 0·026*	184 ± 20	0·18 ± 0·031

* Differs from saline group ($P < 0·001$).
† Differs from saline group ($P < 0·02$).

Tabel 3. *Effect of apomorphine* (25 mg/kg, i.p.) *and pimozide* (2 mg/kg, orally) *on accumulation of catecholamines formed from* [^{14}C]*tyrosine in mouse brain.* [^{14}C]tyrosine was infused i.v. for 20 min starting 40 min after administration of saline or drugs. Immediately after the infusion the animals were killed. Figures represent mean values for groups of 4–6 animals ± s.e.

Treatment	[^{14}C] Dopamine counts/min g^{-1}	[^{14}C] Noradrenaline counts/min g^{-1}
Saline	264 ± 20	111 ± 6
Apomorphine	165 ± 23*	142 ± 18
Saline	348 ± 48	154 ± 16
Pimozide	717 ± 126†	195 ± 12

* Differs from saline group ($P < 0·02$).
† Differs from saline group ($P < 0·01$)

The opposite effects of apomorphine and pimozide on brain dopamine metabolism are of interest with regard to the opposite effects of these drugs on stereotyped behaviour in the rat (Janssen & others, 1968). The selective influence of the two drugs on dopamine synthesis and turnover seems to be more pronounced than that of haloperidol and chlorpromazine since the latter drugs also affect noradrenaline turnover in mouse brain (Carlsson & Lindquist, 1963; Nybäck & others, 1968; Nybäck & Sedvall, 1970).

Persson & Waldeck (1970) found that apomorphine accelerates the disappearance of noradrenaline from mouse brain following synthesis inhibition with α-methyltyrosine. In our experiments apomorphine did not significantly affect synthesis or turnover of [^{14}C]noradrenaline. However, the drug reduced the content of endogenous noradrenaline. Whether apomorphine has a direct or indirect effect on noradrenergic neurons in mouse brain has to be further investigated.

Regarding phenothiazine analogues, we have previously presented evidence that neuroleptic effects are better correlated with changes in dopamine metabolism than with sedative effects that seem to be related to changes in noradrenaline metabolism (Nybäck & others, 1970). The present results support this view, since pimozide, which is a potent neuroleptic drug with negligible sedative properties, markedly accelerated synthesis and turnover of [^{14}C]dopamine but not [^{14}C]noradrenaline.

For generous supply of pimozide, we thank Dr. Oskar Rohte, AB Leo, Hälsingborg.

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Individual elution of noradrenaline (together with adrenaline), dopamine, 5-hydroxytryptamine and histamine from a single, strong cation exchange column, by means of mineral acid-organic solvent mixtures

Few methods have been published which demonstrate the quantitative measurement of the biogenic amines noradrenaline, adrenaline, dopamine, 5-hydroxytryptamine (5-HT) and histamine, and their metabolites, in the same, small sample of tissue after a single extraction and purification procedure. Recently Sadavongvivad (1970) published a technique employing butanol in the organic extractions of catecholamines, and of 5-HT together with histamine, from the same, small sample of tissue. We have developed a column adsorption chromatographical procedure which permits the total amount of each amine, derived from the tissue, to be concentrated into small, individual fractions.

Noradrenaline together with adrenaline has been separated from dopamine on strong cation exchange columns of dimensions 50 mm (in buffer) by 4·2 mm (i.d.). The resin used is Dowex 50W–X4, 200–400 mesh, sodium form (Bertler, Carlsson & Rosengren, 1958, as later modified by Carlsson & Lindqvist, 1962). Noradrenaline, together with adrenaline, is eluted with 8 ml, and dopamine with the following 12 ml, aqueous N HCl. Adopting the procedures of Kahlson, Rosengren & Thunberg (1963) and of Green & Erickson (1964), we were able to elute histamine with 5 ml of aqueous 2N HCl after eluting the catecholamines.

Using large volumes (up to 20 ml) of eluant, 5-HT could be eluted after histamine with 4–6N aqueous HCl or 0·01N aqueous NaOH, the latter being an adaptation of the technique of Wiegand & Scherfling (1962). The eluate volume could be reduced to 4 ml by eluting with 3N ethanolic (50%) HCl, when adopting the procedure of Schildkraut, Schanberg & others (1969).

Whilst the procedure so far described permits the separation of all the amines, with the exception of noradrenaline from adrenaline, some further interesting observations and improvements were made. In common with 5-HT, the elution of noradrenaline, adrenaline and dopamine is also greatly facilitated by the use of certain

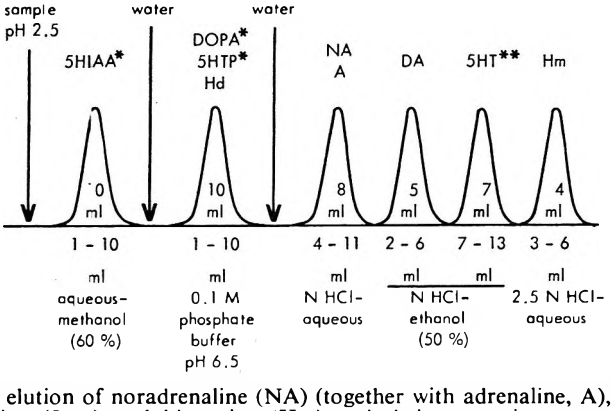

FIG. 1. Order of elution of noradrenaline (NA) (together with adrenaline, A), dopamine (DA), 5-hydroxytryptamine (5-HT) and histamine (Hm) and their respective precursors (dihydroxyphenylalanine–DOPA, 5-hydroxytryptophan, 5-HTP and histidine, Hd) and 5-hydroxyindole acetic acid (5-HIAA), from a strong cation exchange column (of dimensions 72 mm in buffer by 4·0 mm i.d. Dowex[R] 50W–X4, 200–400 mesh, sodium form)

(* According to Lindqvist.)

(** The volume of 5-HT eluate can be reduced to 3·5 ml by eluting with 1·8N HCl-ethylene glycol monoethyl ether (ethyl cellosolve) (50%), whilst still permitting the subsequent, separate elution of Hm.)

Table 1. *Recovery from columns of dopamine* (DA) *and 5-hydroxytryptamine* (5-HT), *when added to rat intestinal extracts**

Sample number	No addition to tissue extract* DA μg	5-HT μg	Addition of amines to respective tissue extract DA μg	5-HT μg	Recovery of added amines DA μg (%)	5-HT μg (%)
1	3·906	—	5·000	—	4·808 (96%)	—
2	9·472	3·256	10·000	3·000	9·405 (94%)	2·530 (84%)
3	3·469	5·426	5·000	5·000	4·517 (90%)	3·980 (80%)
4	0·034	7·145	0·050	6·000	0·053 (107%)	5·155 (86%)
5	13·190	3 719	10·000	3·000	9·889 (99%)	2·657 (89%)
6	3·345	4 317	3·000	5·000	2·714 (90%)	4·184 (84%)
7	isotonic saline solution		1·000	1·000	1·062 (106%)	0·970 (97%)

* Tissue obtained from rats being used in an L-DOPA administration experiment; Atack, Enerbäck & Häggendal—unpublished.

organic solvents mixed with the HCl, but the elution of histamine is almost unaffected.

The procedure which we have adopted as the most promising is represented diagrammatically in Fig. 1. If the sample is loaded onto the column at pH 2·5, the corresponding amino-acids are also adsorbed (Bertler & others, 1958; Wiegand & Scherfling, 1962; Kahlson & others, 1963). Dr. M. Lindqvist found that 5-hydroxyindole acetic acid (5-HIAA) may interfere with the assay of 5-hydroxytryptophan (5-HTP). Consequently, Lindqvist has developed a technique in this laboratory for the elution of 5-HIAA using aqueous methanol (60%) and for 5-HTP, together with dihydroxyphenylalanine (dopa), using 0·1M phosphate buffer, pH 6·5, from a column similar to ours. We have observed histidine to be eluted together with the other amino-acids. The subsequent elution procedure for the amines is unaffected by the methanol and buffer eluants.

The amines (and amino-acids and 5-HIAA) can all be assayed spectrophotofluorometrically. The assays of noradrenaline and adrenaline according to e.g. Bertler & others (1958) or Häggendal (1963), and of histamine according to Green & Erickson (1964), require little modification. Histamine is eluted with 2·5N aqueous HCl because the presence of ethanol or cellosolve was found to lower the sensitivity of the assay. The unlikely possibility of interference by spermidine is being examined. The assay of dopamine by the dihydroxyindole technique has been slightly but significantly modified and specificity tests are being undertaken.

5-HT has been assayed according to the technique of Andén & Magnusson (1967). These authors noted a slow decrease in fluorescence after irradiation, a tendency which appears to be accentuated in the presence of the organic solvents used.

The major changes described above are in the elution patterns of dopamine and 5-HT. In a short experiment to determine the progress of the method, the recoveries of dopamine and 5-HT were recorded and are presented in Table 1.

The procedure as outlined permits the individual separation of the total tissue content of noradrenaline (together with adrenaline), dopamine, 5-HT and histamine (and their respective amino-acid precursors and 5-HIAA). In addition, dopamine is eluted in the much smaller volume of 5 ml (as opposed to 12 ml), without using a stronger acid. Both factors will significantly increase the overall sensitivity of the assay of the amines.

Organic solvents dissolved in aqueous eluting media, to differentially alter the elution behaviour of adsorbed compounds, have previously been used in the separation of inorganic substances. Their successful use in the present study, whereby the elution behaviour of the monoamines, especially 5-HT, was changed but not that of histamine, suggests a wider application to the differential separation of compounds of biological interest.

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Inhibitory effect of propranolol on the vasoconstrictor response to sympathetic nerve stimulation

Propranolol enhances the vasoconstrictor effect of noradrenaline in skeletal muscle (Brick, Hutchinson & Roddie, 1967, Shanks, 1967), spleen (Ross, 1967a) and the mesenteric circulation (Ross, 1967b), an effect thought to be a consequence of inhibition of the effect of noradrenaline on vasodilator β-adrenergic receptors. Whether propranolol, through the same mechanism, may also enhance the vasoconstrictor response to sympathetic nerve stimulation has received less attention. Burks & Cooper (1967) did find propranolol to increase the vasoconstrictor responses both to exogenous noradrenaline and to sympathetic nerve stimulation in canine isolated perfused mesenteric arteries. We have compared the effect of propranolol on the vasoconstrictor responses to peripheral sympathetic nerve stimulation and to intra-arterially injected noradrenaline in the hind leg of the anaesthetized cat.

Cats, 2·5–3·1 kg, were anaesthetized with pentobarbitone sodium, eviscerated, and the lumbar sympathetic chain on one side cut at L3-L4 and a bipolar electrode placed on the distal part of the nerve. The femoral artery on the same side was catheterized in both directions, and the blood flow to the leg passed through a constant-flow Sigmamotor pump. The perfusion pressure to the leg, recorded by means of a Statham transducer on an Offner Dynograph, was initially adjusted to correspond to the systemic arterial pressure. The blood flow to the paw was occluded by means of a tight ligature.

The sympathetic nerves were stimulated for 90 s with impulses of supramaximal voltage, 4 ms duration and a frequency (1–2 impulses/s) (Grass S4 stimulator) that produced an increase of perfusion pressure of 50–80 mm Hg. Nerve stimulations were alternated according to a standardized time schedule with intra-arterial injections of noradrenaline in a dose (0·25–1 μg) that also increased the perfusion pressure 50–80 mm Hg. When stable responses to nerve stimulation and injected noradrenaline had been established, propranolol was infused intravenously during 5 min in a dose of 0·1 mg/kg, followed after 45 min by another infusion of 0·5 mg/kg propranolol. Three responses to each of the vasoconstrictor stimuli were recorded after each dose of

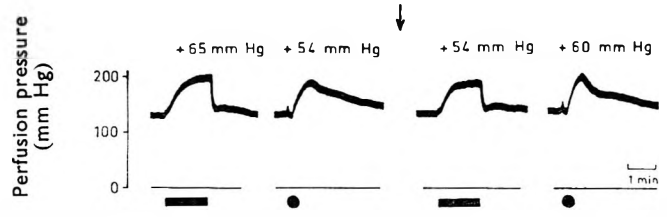

FIG. 1. Influence of (±)-propranolol (0·1 mg/kg, i.v. at arrow) on the effects of electrical stimulation of the lumbar sympathetic nerve (2 impulses/s, 4 ms impulse duration, 6 V shown by bars) and intra-arterially injected noradrenaline (0·5 μg, ●) on the perfusion pressure in a constant-perfused hind leg of an anaesthetized cat.

propranolol. Five experiments were made with (±)-propranolol. In addition five experiments were made in the same way with (+)-propranolol.

Fig. 1 shows typical responses recorded before and after 0·1 mg/kg (±)-propranolol. After this dose the vasoconstrictor response to sympathetic nerve stimulation was consistently reduced (on the average 14 ± 3·7%, $P < 0.025$), while the response to injected noradrenaline was slightly increased or unchanged (average increase 6 ± 3·5%). After 0·5 mg/kg (±)-propranolol the response to noradrenaline was significantly augmented (mean increase 20 ± 6·8%, $P < 0.05$), while the response to nerve stimulation was decreased less than after the lower dose of (±)-propranolol (mean decrease 5 ± 2·8%). (+)-Propranolol, 0·1 and 0·5 mg/kg, elicited on the average no change either of the response to nerve stimulation or of that to injected noradrenaline.

The decreased response to sympathetic nerve stimulation caused by (±)-propranolol concomitant with an unchanged or augmented response to injected noradrenaline indicates that (±)-propranolol reduced the noradrenaline output from the nerve endings. The effect is probably due to β-adrenergic receptor blockade as (+)-propranolol was inactive. The results of cross-circulation experiments, where (±)-propranolol was given exclusively to the leg of the studied animal indicate that the reduced response to sympathetic nerve stimulation was due to a peripheral site of attack.

Our experimental results are not necessarily at variance with those of Burks & Cooper (1967) since these authors reported that after propranolol the vasoconstrictor responses to sympathetic nerve stimulation were less enhanced than those to added noradrenaline. This finding combined with those reported here may indicate that propranolol can influence the vasoconstrictor response to sympathetic nervous stimulation differently, dependent upon whether the reduced release of noradrenaline from the nerve endings or the enhanced sensitivity to it at the smooth muscle cells is of predominant importance for the resultant effect.

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Effects of induced sodium gradients on the transport of metaraminol

Noradrenaline and metaraminol are accumulated by tissues by a process which exhibits saturation kinetics (Iversen, 1963), is Na^+- and K^+-dependent (Iversen & Kravitz, 1966; Gillis & Paton, 1967; Bogdanski, Tissari, & Brodie, 1968; Bogdanski & Brodie, 1969), requires energy (Paton, 1968) and is ouabain-sensitive (Bogdanski & Brodie, 1969). The requirements for amine transport are thus similar to those of the Na^+- and K^+-activated ATPase. Bogdanski & Brodie (1969) have postulated that noradrenaline transport occurs as the result of the operation of a process similar to those proposed by Crane (1965) and Kipnis & Parrish (1965) to account for the transport of sugars and amino-acids by various tissues. According to this model, noradrenaline transport results from an interaction with a membrane carrier the affinity of which for the amine is Na^+-dependent. The carrier transports amine and Na^+ intracellularly where the affinity of the carrier for the amine falls thus releasing amine. Inward noradrenaline transport continues as long as the inward Na^+ gradient is maintained by the extrusion of Na^+ from the cell by the Na^+ pump.

An analysis of this model leads to the predictions that, by driving a net inward Na^+ flux, an electrochemical gradient of Na^+ will drive a net inward flux of amine and that this will continue in the absence of Na^+ pumping as long as an inward Na^+ gradient is maintained (Stein, 1967). The applicability of this prediction to metaraminol transport has been examined by determing the ability of an induced inward Na^+-gradient to produce transport of metaraminol when the Na^+- and K^+-activated membrane ATPase is inhibited.

Male New Zealand white rabbits were killed by a blow on the head after which their hearts were excised rapidly and slices of left ventricle prepared as described previously (Gillis & Paton, 1967; Paton, 1968). Slices were incubated in medium at 37° and gassed with carbon dioxide 5% in oxygen. During a preincubation of 30–60 min all slices were in low Na^+ medium of the following composition (mmol/litre): $NaHCO_3$ 22, NaH_2PO_4 1·2, $MgCl_2$ 1·2, $CaCl_2$ 2·5, Na_2 EDTA 0·03, sucrose 232, KCl 5·0, (+)-glucose 10. Where indicated KCl and (+)-glucose were replaced iso-osmotically with sucrose. Following preincubation slices were placed in either low Na^+ medium or a normal Na^+ medium in which sucrose was replaced by NaCl 116 mmol/litre, [^{3}H]-metaraminol added to achieve a final concentration of 2×10^{-8}M of the free base and the incubation continued for a further 6 or 30 min. During preincubation and incubation with [^{3}H] metaraminol the Na^+ pump was inhibited by either removal of K^+ or 10^{-5}M ouabain or low temperature (4°) or metabolic inhibition; full details are given in Table 1. The slices were then digested using NCS Solubiliser (Amersham Searle) and the content of [^{3}H]amine measured as described previously (Paton, 1968). Retention of [^{3}H]metaraminol was expressed as a ratio (R) calculated by dividing the [^{3}H]disintegrations/min g^{-1} of slices by [^{3}H] disintegrations/min ml^{-1} of medium. Chromatographically pure (±)-metaraminol-7-[^{3}H]-hydrochloride with a specific activity of 6·5 Ci/mmol was obtained from the New England Nuclear Corporation.

The results obtained are shown in Table 1. Slices were preincubated in low Na^+ medium to prevent Na^+ enrichment which would have otherwise occurred since the Na^+ pump was inhibited. Uptake of [^{3}H] metaraminol was measured after 6 min as well as 30 min because Eddy, Mulcahy & Thomson (1967) have shown that under similar conditions the maximal uptake of glycine by ascites-tumour cells occurred within 10 min, the subsequent decline in uptake of glycine apparently being produced by a rise in intracellular Na^+ with a consequent reduction in the inward Na^+ gradient. It can be seen that when the Na^+ pump was not inhibited, the effects of low Na^+

Table 1. *Influence of Na^+ gradient on the uptake of* [^{3}H]*metaraminol by rabbit heart slices*

Group	Preincubation	Incubation with [^{3}H] metaraminol	R value (mean ± s.e.) 6 min	30 min
A	Low Na^+ medium (30 min)	Low Na^+ medium	1·86 ± 0·27 (12)	3·52 ± 0·32 (18)
		Normal Na^+ medium	2·73 ± 0·26 (12)	8·15 ± 0·59 (18)
B	Low Na^+ medium plus 10^{-5}M ouabain (30 min)	Low Na^+ medium plus 10^{-5}M ouabain	1·20 ± 0·13 (6)	2·47 ± 0·10 (7)
		Normal Na^+ medium plus 10^{-5}M ouabain	1·13 ± 0·13 (6)	1·75 ± 0·07 (9)
C	Low Na^+, K^+ free medium (60 min)	Low Na^+, K^+ free medium	1·58 ± 0·10 (8)	3·49 ± 0·21 (8)
		Normal Na, K^+ free medium	1·20 ± 0·16 (8)	1·97 ± 0·13 (8)
D	Low Na^+ medium at 4°	Low Na^+ medium at 4°	0·68 ± 0·04 (6)	1·18 ± 0·10 (6)
		Normal Na^+ medium at 4°	0·68 ± 0·16 (6)	0·96 ± 0·07 (6)
E	Low Na^+, glucose free medium (60 min) with 10^{-3}M dinitrophenol (40 min) and 10^{-3}M iodoacetic acid (20 min).	Low Na^+ glucose free	1·09 ± 0·04 (10)	1·79 ± 0·07 (10)
		Normal Na^+ glucose free	0·96 ± 0·08 (10)	1·55 ± 0·07 (10)

Number in brackets is no. of slices in each group.

medium on uptake were rapidly reversible, an increase in Na^+ gradient resulting in an immediate increased uptake of [^{3}H] metaraminol (Group A). However when the Na^+ pump was inhibited by either 10^{-5}M ouabain (Group B), absence of external K^+ (Group C), low temperature (4° C) (Group D) or metabolic inhibition (Group E), an increase in the Na^+ gradient did not increase the uptake of [^{3}H]metaraminol after either 6 or 30 min incubation. An inward directed Na^+ gradient similarly failed to increase the transport of [^{3}H]noradrenaline into synaptosomes pretreated with ouabain (Tissari, Schönhöfer & others, 1969) or metabolic inhibitors (White & Keen, 1970).

An analysis of the proposed model for noradrenaline transport also leads to the prediction that an induced outward Na^+ gradient should produce transport of noradrenaline out of the cell against the concentration gradient as has been reported for sugar transport by intestine (Crane, 1965). This phenomenon could not however be demonstrated for metaraminol transport using ventricular slices obtained from animals pretreated with reserpine, 2 mg/kg intravenously 24 h previously. Slices were first incubated in normal Na^+ medium containing [^{3}H]metaraminol for 3 min and then transferred to a Na^+-free medium containing [^{3}H]metaraminol for an additional 10 or 20 min. The R values obtained were 1·15 ± 0·11 (12) at 3 min, 1·62 ± 0·06 (9) at 13 min and 1·82 ± 0·04 (10) at 23 min.

The findings outlined here together with those recently reported by Tissari & others, (1969) and White & Keen (1970) have shown that an inwardly directed Na^+-gradient could not serve as the only driving force for the transport of noradrenaline and metaraminol. Similarly an outwardly directed Na^+-gradient did not reverse the direction of amine transport. It thus seems likely that the mechanism for noradrenaline transport postulated by Bogdanski & Brodie (1969) requires modification. The activity of the Na^+- and K^+-activated ATPase appears essential for noradrenaline and metaraminol transport and may be required to provide energy for transport or to phosphorylate the amine carrier.

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Reduced variation of [^{3}H]noradrenaline uptake into rat submaxillary glands by atropine pretreatment

The submaxillary gland of the rat is useful for the study of peripheral adrenergic mechanism. It has a rich adrenergic innervation with a mean noradrenaline content of about 1·2 μg/g tissue, is paired so that it is always possible to get a matched control, and is easily accessible for different mechanical manipulations. The adrenergic nerves of the gland, with cell bodies in the superior cervical ganglion, and the preganglionic fibres in the cervical sympathetic trunk, are easily reached from the neck.

In the studies made in this laboratory using the preparation a serious problem was encountered in the great variation between different animals in the uptake of labelled noradrenaline (^{3}H-NA) or its analogues. A possible explanation for this could be that the secretory activity of the submaxillary gland and thus the blood flow through the organ varied widely between and within animals. The following experiment was made to see if a uniform increase or decrease of the secretory activity of the submaxillary gland could reduce the differences in uptake of labelled amines.

Male Sprague-Dawley rats, 170–270g, kept at 31°, received 1 μg/kg ^{3}H-NA (specific activity 8·45 Ci/mmol, NEN Chemicals) in a tail vein. One group of 8 rats was pretreated with atropine, 1 mg/kg intraperitoneally 30 min before the ^{3}H-NA injection. In another group, 8 rats were forced to chew dry wheat starch 5 min before and 5 min after the ^{3}H-NA injection. A third group, also of 8 rats, was not pretreated. Three h after the ^{3}H-NA administration the rats were killed. The hearts and the submaxillary + sublingual glands were immediately taken out, weighed and homogenized in ice-cold 0·4 N perchloric acid. The salivary glands from both sides were analysed together. Noradrenaline was separated on cation exchange columns and the tritium contents of the eluates were measured by liquid scintillation counting (Carlsson & Waldeck, 1963; Stitzel & Lundborg, 1966).

Three h after the ^{3}H-NA injection a mean 1·58 ng/g (variance 0·297) ^{3}H-NA was found in the submaxillary + sublingual glands. Neither after pretreatment with atropine (1·41 variance 0·041) nor with dry wheat starch (1·50 variance 0·304) was this

amount significantly changed. The variance of the observations, however, was significantly reduced by atropine pretreatment ($P < 0.01$). The hearts of the control rats contained a mean 4·58 ng/g of ^{3}H-NA 3 h after the injection and about the same in the rats pretreated with dry wheat starch (4·56). After atropine pretreatment, the content of ^{3}H-NA in the hearts (5·52) was significantly increased ($P < 0.001$). The variance of the observation was not changed by pretreatment. As alterations in body temperature might influence the results, rectal temperature was recorded immediately before killing, and did not differ between groups.

Atropine, 1 mg/kg, completely abolishes the secretory response of the submaxillary gland by parasympathetic stimulation in the rat (Ohlin, 1965). Also the sympathetic nerves can mediate reflexly evoked secretory responses, although these are comparatively small (Ohlin, 1968). It is thus probable, that the secretory activity of the glands after atropine treatment is less variable than in the glands of the control rats. As the blood flow through the gland is correlated with the secretory activity (see Burgen & Emmelin, 1961), the variations in blood flow should also be reduced by atropine.

The uptake into an organ of exogenously administered noradrenaline is dependent on two major factors: the density of the adrenergic nerve terminals and the blood flow through the organ (Kopin, Gordon & Horst, 1965). In the present experiment reduced variations in blood flow through the glands is the most probable explanation for the smaller variance of the ^{3}H-NA values found after atropine.

Chewing dry wheat starch increases the salivary secretion (Hillarp, 1949). This treatment, when performed 5 min before and 5 min after the ^{3}H-NA injection, did not change the variation of the ^{3}H-NA content of the glands. Probably the stimulation was not maximal and perhaps not equally strong in all rats giving variations in blood flow and in uptake of ^{3}H-NA.

If atropine reduces and starch chewing stimulates secretory activity and blood flow, a decrease in ^{3}H-NA uptake by the salivary gland after atropine and an increase after starch chewing would be expected. Contrary to this, the average uptake was not influenced by these procedures; possibly other factors influencing ^{3}H-NA uptake are simultaneously affected.

Atropine pretreatment increased the content of ^{3}H-NA found in the hearts. Increased heart rate and blood flow through the heart, leading to a greater uptake of administered ^{3}H-NA, could account for this difference.

The results of this experiment indicate that atropine pretreatment can diminish uptake by the submaxillary glands by reducing variations in secretory activity and blood flow. When using labelled substances in the study of adrenergic transmission in the submaxillary glands, such pretreatment can be valuable.

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The binding of neurohypophysial hormones in van Dyke protein

Since the isolation of the carrier protein (van Dyke protein) containing oxytocin and vasopressin from the ox posterior pituitary gland (van Dyke, Chow & others, 1942) the nature of the binding of these hormones to the protein has been thought to be a loose one involving the amino-group of the hormones (van Dyke, 1968). In addition, oxytocin and lysine vasopressin can be inactivated by exposure to acetone which is thought to act on this amino-group (Yamashiro, Havran & others, 1965; 1967). The following experiments add to the evidence presented on the nature of the binding of these hormones to their carrier protein.

van Dyke protein was subjected to dialysis, boiling before dialysis, and to gel filtration through Sephadex G-25 (fine bead) (Sawyer, Freer & others, 1967) in a column 2·5 cm diameter and 100 cm long, at a flow rate of 1·33 ml/min in attempts to separate the hormones from the protein. Acetic acid eluates were collected in 6 ml samples in a fraction collector. van Dyke protein was also subjected to exposure to acetone for 16 or 65 h, without boiling, then after boiling for 2½ h, with vasopressor activity monitored. For comparison, synthetic arginine vasopressin (Sandoz) was similarly subjected to acetone treatment, and the time sequence for the "regeneration" of activity by boiling was also noted. Protein was measured spectrophotometrically by the Folin-Lowry method, oxytocic activity by the rat uterus method of Munsick (1960) in magnesium free van Dyke and Hastings solution, and vasopressor activity by changes in the blood pressure of the dibenzylene pretreated rat (Dekanski, 1952).

The neurohypophysial hormones were easier to separate from their carrier protein than was thought. Simple dialysis for 16 h reduced the vasopressor activity of the protein by 40%. Boiling before dialysis increased the reduction to 52%. Boiling alone reduced the potency by 29%.

Gel filtration separated the hormones completely from the carrier protein. 90% of the weight of the protein was recovered, but this contained only 0·06% of its original vasopressor and oxytocic activities. The hormones could also be separated from each other, and 90% of both oxytocic and vasopressor activities were recovered.

Acetone treatment did not affect the vasopressor activity in van Dyke protein, but boiling to liberate the hormones before acetone treatment reduced the vasopressor activity by 40%. The activity was "regenerated" by boiling in acetic acid. In contrast, synthetic arginine vasopressin was almost completely inactivated by exposure to acetone, when 98% of the potency was lost (Table 1), but regained 86% of its vasopressor activity after boiling in 0·002M acetic acid for about 15 min.

Table 1. *Effect of acetone on the vasopressor activity of van Dyke protein and arginine vasopressin*

Treatment	Vp activity van Dyke protein U/mg	%	Vp activity arginine vasopressin	%
Control	16·7 ± 1·7*	100	0·48 U/ml	96
Acetone (65 h)	14·2 ± 1·4	85	0·01 U/ml	2
Acetone (16 h)	17·3 ± 1·8	102		
Boiled before acetone treatment (65 h)	10·2 ± 1·4	61		
Boiled before acetone (16 h)	10·3 ± 1·5	61		
Regenerated acetone powder (16 h)	13·3 ± 1·2	80		
Regenerated acetone powder (65 h)	14·7 ± 1·6	88	0·33 U/ml	66

* Limits of error of estimations.

The binding of the neurohypophysial hormones to their carrier protein seems to be loose as they can be separated not only by electrophoresis and counter-current distribution (van Dyke, 1968) but also by gel filtration, dialysis and boiling. Acetone did not inactivate the vasopressor activity of van Dyke protein as the hormone was still bound to the carrier protein. But a simple procedure like boiling which liberated about 30% of the hormone, exposed this amount to the action of acetone, and reduced potency by 40%; the extra 10% was probably inactivated before it could re-establish binding with protein. The inference is that acetone acts on the same amino-group with which the hormone attaches itself to the carrier protein.

The author is grateful to Dr. H. B. van Dyke for advice and for generous supplies of van Dyke protein (Batch No. 1090 C), and to Mr. A. Ganesan for technical assistance.

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Inhibition by *p*-chloroamphetamine of the conversion of 5-hydroxytryptamine to 5-hydroxyindoleacetic acid in rat brain

p-Chloroamphetamine (PCA) causes a lowering of 5-hydroxytryptamine (5-HT) in whole brain of rats (Pletscher, Bartholini & others, 1964; Fuller, Hines & Mills, 1964, 1965). Two possible mechanisms are, the inhibition of 5-HT synthesis, and the release of 5-HT from binding sites in brain. The fact that PCA also lowers 5-hydroxyindoleacetic acid (5-HIAA) concentrations in brain pointed to the first possibility, since releasing agents make 5-HT susceptible to attack by monoamine oxidase and thus raise the 5-HIAA concentration (Roos & Werdinius, 1962). An alternative explanation for the lowered 5-HIAA concentration was proposed (Fuller, 1966) on the basis of the ability of PCA to inhibit the oxidation of 5-HT by brain mitochondria from rats. Based on the *in vitro* potency of PCA as an inhibitor and the concentrations found to be present in the brains of rats given PCA, we suggested that the conversion of 5-HT to 5-HIAA in rat brain might be inhibited. To provide direct evidence for this possibility, we have now examined the metabolism of [^{3}H]5-HT formed from [^{3}H]5-hydroxytryptophan (5-HTP) in rats treated with PCA.

In these experiments, male albino rats, about 150 g, were injected intraperitoneally with saline or with PCA at a dose of 20·6 mg/kg (0·1 mmol/kg). 16 h later the rats were given an intraperitoneal injection of [^{3}H]DL-5-HTP (generally labelled, from Volk Radiochemical Company). A tracer amount of the 5-HTP was injected

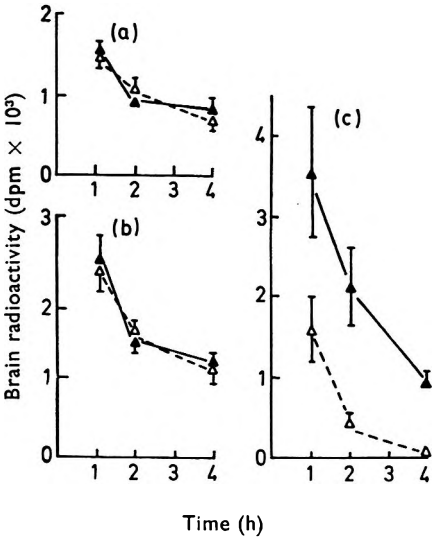

FIG. 1. Radioactive 5-HTP (a), 5-HT (b) and 5-HIAA (c) in whole brain of rats given an i.p. injection of [³H]DL-5-HTP at zero time. Solid lines represent control rats, broken lines are for rats given PCA 16 h before the 5-HTP injection. Means and s.e. for 3 rats per group are shown.

(0·33 mCi/kg; specific activity was 9·1 mCi/mg). Rats were killed by decapitation in groups of three 1, 2 and 4 h later. The brains were rapidly removed and frozen on dry ice. Radioactive metabolites were separated according to the methods of Feldstein & Wong (1965) into fractions containing 5-HTP, 5-HT and 5-HIAA. Radioactivity in each fraction was determined by liquid scintillation spectrometry.

The results are in Fig. 1. At all three time intervals, the amounts of radioactivity present as 5-HTP and as 5-HT were alike in the control and PCA-treated groups. However, the amount of radioactivity present as 5-HIAA was markedly decreased, the differences at the 2 and 4 h intervals being statistically significant ($P < 0.05$). These data strongly suggest that the conversion of radioactive 5-HT to 5-HIAA was decreased and provide direct evidence that inhibition of monoamine oxidase can occur in PCA-treated rats.

Recently, Sanders-Bush & Sulser (1969) have published data indicating that PCA may inhibit the hydroxylation of tryptophan in brain and thus inhibit 5-HT synthesis. Our data do not contradict that possibility.

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A simple method for the simultaneous recording of blood pressure and heart rate

Blood pressure and heart rate can be simultaneously and accurately recorded, over a wide pressure range, using a single pressure transducer input incorporated into the circuit outlined in Fig. 1.

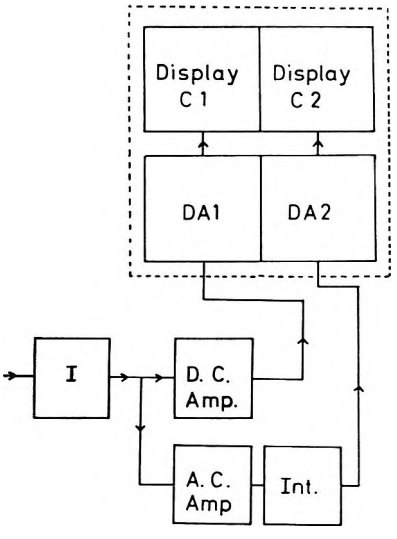

FIG. 1. Schematic diagram of the apparatus used for the simultaneous measurement of blood pressure and heart rate.
I = Input source: a pressure transducer (Bell and Howell: Type 4-326-L212). D.C. Amp = a blood pressure amplifier ('Devices' Type D.C.2D). A.C. Amp = an A.C. amplifier ('Devices' Type A.C.7). Int = a rate meter ('Devices' Type 2751). D.A.1 and D.A.2 = driver amplifiers for channels 1 and 2. Displays C1 and C2 = display channels 1 and 2.

The equipment shown in broken outline represents the components of a Devices (M2 type) two channel pen recorder fitted with D.C.5 pen driver amplifiers.

Because the A.C.7 amplifier is not used as a differential amplifier, a shorting link must be placed between pins 1 and 2 of the 3 way jack plug which is inserted in the "signal input 1" jack socket (see diagram of input signal paths, Fig. 7, 46 Devices, M2R Manual).

Description of system. The pulse pressure impulses are converted, by means of the input transducer, into an electrical signal which is then used as the primary signal for activating both the pressure amplifiers and the rate meter. To record heart rate, the signal is passed into the amplifier, and from thence into the integrator before being fed into the second channel of the pen recorder. Blood pressure is displayed on Channel 1.

If pulse pressures are low, the signal will be insufficient for feeding directly into the integrator and this will fail. Therefore, a means of amplifying the pulse signal is essential and for this reason the A.C.7 amplifier is incorporated into the system. This amplifier also has useful time constant and filter facilities for exclusion of unwanted signal components.

Thus simultaneous blood pressure and heart rate measurement and recording can be made and, unlike ECG-actuated heart rate meters, distortions and artifacts which may arise during electrical stimulation of the preparation are isolated.

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Antagonism by methysergide of the 5-hydroxytryptamine-like action of toxic doses of fenfluramine in dogs

Fenfluramine produces anorexia in experimental animals and in man, but does not stimulate the central nervous system (Le Douarec, Schmitt & Laubie, 1966; Colmore & Moore, 1968). It has been reported to cause depletion of catecholamines both centrally and peripherally (Ziance & Kunnard, 1968; Duce & Gessa, 1966) and in contrast to amphetamine to release brain 5-hydroxytryptamine (Opitz, 1967; Duhault & Verdavainne, 1967). Also in contrast to amphetamine it lowers body temperature in experimental animals (Jespersen, Bonaccorsi & Garattini, 1969; Bizzi, Bonaccorsi & others 1969).

The effect of fenfluramine alone and in combination with methysergide (a specific 5-HT antagonist) was studied in 5 male beagle dogs by recording the rectal temperature and observing gross behaviour. (±)-Fenfluramine hydrochloride or saline was given subcutaneously after recording of the basic temperature, and 2½ h later methysergide bimaleate was administered intraveneously. Each dog was tested 3 times at 7–10 day intervals.

Fenfluramine causes hypothermia in dogs—as in other species—with maximum effect 2½ h after injection. Methysergide given alone does not influence body temperature at the dose tested, but when given 2½ h after fenfluramine a clear-cut and statistically significant antagonism of the fenfluramine-induced hypothermia was revealed. [$P < 0.05$, $P < 0.01$ and $P < 0.01$ at 3, 3½ and 4 h respectively (Student's *t*-test)].

Fenfluramine caused changes in behaviour similar to those reported by Bogdanski, Weissbach & Udenfriend (1958) after injection to dogs of 5-HTP, a 5-HT precursor, which at decarboxylation raises the brain level of 5-HT up to 10 times (Udenfriend, Weissbach & Bogdanski, 1957). The fenfluramine-induced reactions were: sedation, mydriasis, apparent blindness, whining by petting, diarrhoea and unwillingness to keep still during measurement of rectal temperature. All these symptoms were more or less improved after methysergide. Two of the dogs behaved quite normally half an hour after the injection, while the other 2 dogs were still partially sedated. The fenfluramine controls continuously showed the mentioned symptoms up to 5–6 h after administration.

Some evidence was obtained that methysergide also antagonized the fenfluramine-induced anorexia, because when food was presented 1 h after the administration of methysergide or saline, none of the 4 dogs treated with fenfluramine + saline showed any interest in the food, while 2 of the 4 dogs treated with the combination ate with fairly good appetite, and 1 dog showed interest without eating.

The antagonism of subcutaneously administered methysergide (0·05 mg/kg) to the fenfluramine sedation and discomfort was confirmed in an experiment with 4 female beagles. The antagonism judged on gross behaviour observations was evident in 3 of these dogs which had received 2·5, 5 or 20 mg/kg fenfluramine subcutaneously about 3 h before. The behaviour of 1 dog given fenfluramine 5 mg/kg was not improved by methysergide.

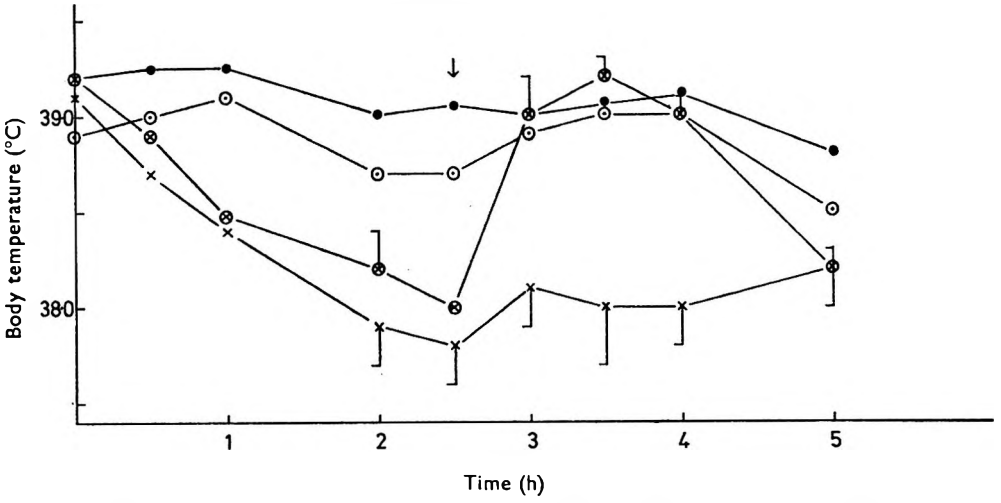

FIG. 1. The effect of fenfluramine alone or in combination with methysergide on the temperature in dogs

At 0 h subc.	*At 2½ h i.v.*	
Saline	Saline	—·— (4 dogs)
Saline	Methysergide 0·05 mg/kg	—⊙— (3 dogs
Fenfluramine 20 mg/kg	Saline	—×— (4 dogs)
Fenfluramine 20 mg/kg	Methysergide 0·05 mg/kg	—⊗— (4 dogs)

At ↓ methysergide or saline was injected. Vertical bars indicate standard error of the mean.

In summary a large dose of fenfluramine given to dogs caused symptoms which were grossly similar to those reported after administration of 5-HTP, indicating that 5-HT plays an important role in the reactions. The symptoms improved after administration of the 5-HT antagonist methysergide, which also seemed to antagonize the fenfluramine-induced anorexia.

Methysergide might be of value in the treatment of fenfluramine overdosage.

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Potentiation by amantadine hydrochloride of L-dopa-induced effects in mice

Recently a therapeutic effect of the antiviral agent amantadine in Parkinsonism has been reported (Schwab, Poskanzer & others, 1969; Millac, Hasan & others, 1970). Since it is generally recognized that L-dopa is effective in Parkinsonism, it is of interest that administration of amantadine to Parkinsonian patients receiving L-dopa lowers the dose of L-dopa required to maintain the effect (Schwab, 1969). Therefore we have investigated the effect of amantadine hydrochloride on L-dopa-induced effects on motor activity and gross behaviour in mice.

Female NMRI mice, about 20 g, were injected intraperitoneally with L-dopa in doses ranging from 75–1000 mg/kg. Some of the animals received amantadine hydrochloride 100 mg/kg 95 min before L-dopa. Control mice were treated with saline 0·6 ml or amantadine hydrochloride, 100 mg/kg, and 95 min later saline 0·6 ml all intraperitoneally. Measurements of motor activity were made on groups of three mice by means of two sets of Animex activity meters, (Svensson & Thieme, 1969). Recordings were made for 10 min beginning 25 min after the injection of L-dopa or saline and 20 min after placing the animals in the test cages.

Amantadine 100 mg/kg did not exert any influence of its own on motor activity. Vernier, Harmon & others, (1969), also found that only high doses of amantadine caused a moderate increase in motor activity. Neither did we find any change in the general appearance of the animals. Pretreatment with amantadine did cause a potentiation of the L-dopa effects on motility (Fig. 1). The dose curve of L-dopa after amantadine pretreatment parallels that of L-dopa alone. There was no significant difference between the slopes of the corresponding linear parts of the curves. After amantadine about half the amount of L-dopa was required to obtain the same effects as those of L-dopa alone.The potentiation is of the same magnitude as that caused by MK 485, a peripheral dopa decarboxylase inhibitor [α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl) propionic acid] (Strömberg, 1970). However, in contrast to MK 485, amantadine also appeared to potentiate the peripheral effects of L-dopa.

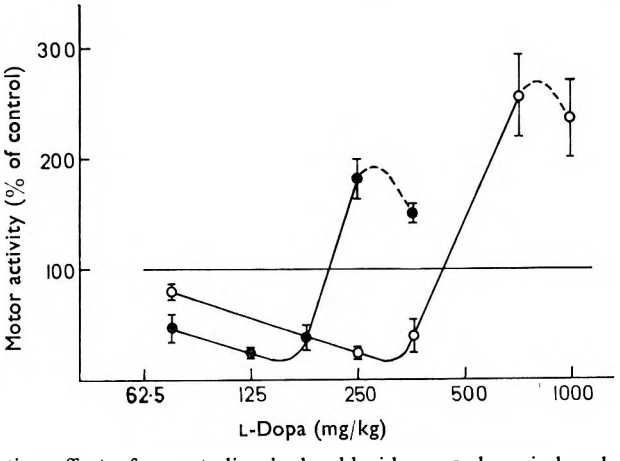

FIG. 1. Potentiating effect of amantadine hydrochloride on L-dopa induced changes in motor activity in mice. Shown are the means ± s.e. expressed as % of the respective control values. These were 534 ± 106 and 647 ± 93 for saline (0·6 ml) and amantadine hydrochloride (100 mg/kg) respectively. Each point represents the mean activity of four groups consisting of three mice. Recordings were made during 10 min beginning 25 min after the injection of L-dopa or saline and 120 min after amantadine. All injections were given i.p. ●—● Amantidine HCl 100 mg/kg + L-dopa. ○—○ L-Dopa.

Thus it is unlikely that amantadine acts by inhibiting dopa decarboxylase in the peripheral sympathetic system.

Animals receiving amantadine and L-dopa, 250 mg/kg, were less jerky than those given L-dopa 700 mg/kg and showed a general appearance similar to that seen after treatment with apomorphine and clonidine by Andén, Corrodi & others, (1970).

Some of the animals receiving L-dopa, 350 mg/kg, after amantadine died in convulsions within 2 h. Similar convulsions, although less pronounced, were also seen in a few mice given L-dopa alone at 1000 mg/kg. This probably explains the reduction in motor activity found in the animals at these doses.

We thank Geigy Ltd, Switzerland for amantadine hydrochloride (Symmetrel). The technical assistance of Miss Barbro Jörblad is gratefully acknowledged.

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Intestinal blood flow and absorption of non-dissociable substances

Experiments concerning the influence of intestinal blood flow on absorption of drugs are rare. Ochsenfahrt & Winne (1969) have shown that the appearance of aniline, amidopyrine, antipyrine, benzoic and salicylic acid in the intestinal venous blood of rats is dependent on blood flow. These findings are now complemented by data of the following non-dissociable substances: tritiated water, methanol, ethanol, urea, ethylene glycol, glycerol, erythritol, and ribitol. Jejunal loops (6–10 cm) of urethane-anaesthetized and heparinized rats were perfused by an isotonic phosphate buffer (pH 7), or by Ringer solution containing the substances. The jejunal vein of the loop was punctured and the outflowing blood collected and weighed (= blood flow). The lost blood was substituted by an infusion of heparinized rat blood into the jugular vein. The intestinal blood flow was changed from high to low and from low to high values by varying the blood infusion. A drop recorder in the venous outflow provided a control record. The concentrations of the [^{14}C]-labelled substances and of tritiated water were measured in the collected blood. Appearance rate and blood flow are referred to wet tissue weight (Ochsenfahrt & Winne, 1969).

The results are summarized in Fig. 1. The figures are corrected to a concentration of 50 nmol/ml in the perfusion solution. Tritiated water showed the highest appearance rate which was almost strictly dependent on blood flow. The absorption rate and its dependence on blood flow decreased in the order ethanol, methanol, glycerol, ethylene glycol, urea, erythritol, and ribitol. The absorption of ribitol is independent of blood flow.

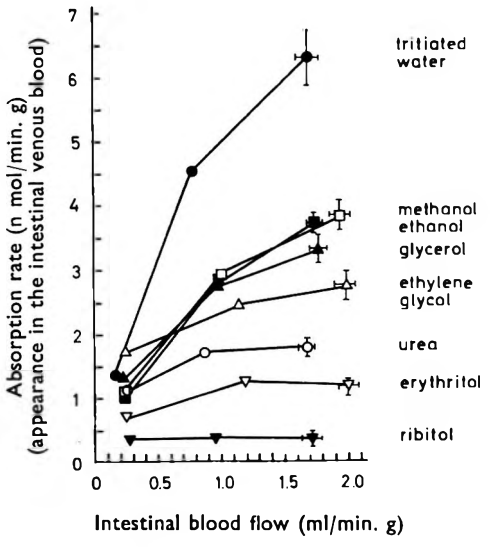

FIG. 1. Dependence of intestinal absorption on blood flow. Luminal perfusion of jejunal loops of rats. Data corrected to a concentration of 50 nmol/ml in the perfusion solution. 95% confidence limits.

For the theoretical interpretation of the curves a simplified equation can be derived from a three-compartment-model (Winne & Ochsenfahrt, 1967):

$$\phi = \frac{C_{DO} - C_{PA}}{\frac{1}{k\ F_D} + \frac{1}{\alpha a_1 V_B}}$$

ϕ = appearance rate in the intestinal venous blood (mol/min), C_{DO} = concentration in the luminal perfusion solution (mol/ml), C_{PA} = arterial plasma concentration (mol/ml), k = permeability coefficient of the epithelium (ml min^{-1} cm^{-2}), F_D = mucosal surface area (cm^2), α = fraction of blood flowing through capillaries near the epithelium, V_B = whole intestinal blood flow (ml/min), a_1 = concentration ratio blood to plasma. The denominator can be interpreted as resistance of the region between the intestinal lumen and the blood. The whole resistance is divided into two parts (first and second term of the denominator): (1) resistance of the region between the intestinal lumen and the capillary wall (mainly the resistance of the epithelium), (2) resistance of the drainage system. A high permeability of the epithelium means a small first term of the denominator, the second term with the blood flow determines the absorption rate. The appearance in the intestinal venous blood is blood flow-limited (example: tritiated water). A low permeability of the epithelium means a large first term of the denominator, the second one can be neglected. The absorption rate is independent of blood flow (example: ribitol).

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Alteration of rat skin acid and alkaline phosphatase and β-glucuronidase activity after topical application of fluocinolone acetonide cream

The evaluation of topical anti-inflammatory agents presents some perplexing problems because there is a lack of an appropriate assay procedure; an inability to avoid ingestion without creating stressful conditions; physiological differences between rat and human skin, and difficulty in separating local and systemic effects. The present investigation is an attempt to evaluate and resolve some of these problems.

Male Sprague-Dawley rats (150–160 g) were individually housed and had free access to tap water and rat chow. A pouch was formed caudal to the interscapular region by a subcutaneous injection of air (25 ml). The air pouch served as the site for drug application and as a physical barrier to avoid drug ingestion. The rats were divided into four groups of 20 each and pouch skin samples were obtained from: untreated control rats; shaved skin controls; shaved skin treated with a control cream; shaved skin on which one half of the area received a control cream (D-1) and the other half (D-2) received a cream* containing fluocinolone acetonide, 0·025%. The active preparation (100 mg) or control cream (or both) was applied daily to an area approximately 1 inch square. Ten animals from each group were killed at 12 h and 10 at 4 days. A section of skin over the air pouch was weighed on an analytical balance and placed between two blocks of dry ice for 1 min. The frozen skin was placed in a rubber finger cot and shattered by hitting it with a hammer. The tissue was transferred to a flask containing 10 ml of cold distilled water and was homogenized with a VirTis No. 45 homogenizer until a uniform suspension was obtained (approximately 5 min). The homogenate was diluted to a suitable concentration and aliquots were used for the assay of β-glucuronidase (Talalay, Fishman & Huggins, 1946) and acid (Manning, Babson & others, 1966) and alkaline phosphatase activity (Manning, Butler & others, 1967). Dry weights were obtained by drying pieces of skin under vacuum at 60° for 24 h. The mean and standard error was calculated for all groups and the significance of the differences between control and treated groups was determined by the Student's *t*-test.

The separately determined enzyme levels of the adjacent areas of shaved rat skin treated with the control and active creams (Group D-1 and D-2) were significantly decreased at 12 h and at 4 days when compared with the untreated control Groups A and B, and, except for the acid phosphatase at 4 days, with Group C (Table 1). The acid phosphatase activity on day 4 for Group C (shaved + control cream) was lower than its 12 h value and equal to the shaved rat skins receiving the control and active creams at both time periods All groups had significantly lower alkaline phosphatase levels on day 4 than at 12 h. The shaved-skin controls (Group B) had a reduced β-glucuronidase activity on day 4.

This investigation has established that a topically applied formulation depressed the acid and alkaline phosphatase and β-glucuronidase activity in rat skin. Since the altered enzyme activities occurred equally in separate skin areas of the same animal, whether treated with the control or active creams, a definite systemic effect was apparent. The air pouch alone significantly depressed the alkaline phosphatase at day 4 in all groups; thus the enzyme levels at this time for Groups D (shaved + control and active cream) reflect the combined effect of the active cream and the formation of an air pouch. In contrast, shaving reduced the β-glucuronidase reaction, while the control cream appeared to lower the acid phosphatase in Group C

* Synalar: Syntex Laboratories, Palo Alto. The control areas were prepared to be similar to the active preparation.

Table 1. *Acid phosphatase, alkaline phosphatase and β-glucuronidase levels in rat skin of four groups of* 20 *rats*

Group	Acid phosphatase[1]		Alkaline phosphatase[2]		β-Glucuronidase[3]	
	12 h	4 Days	12 h	4 Days	12 h	4 Days
A. Untreated ..	30,189 ±1674	29,543 ±2231	45,512 ±3945	32,132 ±2616§	7906 ±442	7969 ±243
B. Shaved ..	34,731 ±1138	35,892 ±2355	48,627 ±3429	29,835 ±1295§	8982 ±340	7927 ±260§
C. Shaved control cream ..	33,590 ±1858	18,943 ±1920*†§	41,959 ±3496	29,662 ±2603§	7812 ±313†	7055 ±380
D-1. Shaved control cream .. +	21,056 ±2250*†‡	20,004 ±2432*†	19,247 ±1995*†‡	11,575 ±1295*†‡§	5639 ±339*†‡	4848 ±277*†‡
D-2. Shaved, active cream ..	23,639 ±2889†‡	17,356 ±1837*†§	23,275 ±2347*†‡	13,991 ±859*†‡§	6543 ±307*†‡	5136 ±184*†‡§

* Significantly different from untreated control, Group A.
† Significantly different from shaved control, Group B.
‡ Significantly different from control cream, Group C.
§ Significantly different from 12 h.

[1] One unit of acid phosphatase activity liberates 1 μg of α-naphthol from sodium α-naphthyl acid phosphate in 30 min at pH 5·2 and 37° under standard conditions.
[2] One unit of alkaline phosphatase activity liberates 1 μg of phenolphthalein in 30 min from phenolphthalein monophosphate at pH 10·1 and 37° under standard conditions.
[3] One unit of β-glucuronidase activity liberates 1 μg of phenolphthalein in 30 min from phenolphthalein monoglucuronide at pH 4·5 and 37° under standard conditions.

(shaved + control cream) on day 4. This latter effect suggests that cream bases cannot be considered biologically inert.

Since fluocinolone acetonide decreased acid phosphatase and β-glucuronidase, the presented data support the hypothesis that anti-inflammatory agents stabilize the lysosomal membrane (DeDuve, Wattiaux & Wibo, 1962; Weissman & Thomas, 1964; Weissmann, 1965). It is difficult to envisage that any lysosomes, if present, could possibly resist the drastic physical treatment of the tissue samples described. A possible alternative explanation for the decreased hydrolase levels may be that cortisone and its derivatives may directly, or indirectly, partially depress protein-enzyme synthesis resulting in fewer lysosomes (also endoplasmic reticulum and Golgi vesicles) being produced.

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Inhibition by propranolol of ethanol-induced narcosis

Large doses of propranolol increase the sleeping time of mice or rats injected with barbiturates (Leszkovsky & Tardos, 1965) or chloral hydrate (Laverty & Taylor, 1968). This action is attributed to the central depressant property, rather than the β-blocking effect of propranolol. Ethanol also depresses the central nervous system. When biogenic amines are given with ethanol the sleeping time of mice is prolonged (Rosenfeld, 1960) and the lethal effect of large doses of amine is potentiated. We now report on the influence of propranolol on the depressant effect of ethanol.

Female, Swiss-Webster mice, 20–30 g in groups of 5, were tested for each dose; controls numbered 10 or more. Only 5 mice were observed at each time and observation was continuous. The drugs propranolol, 1 mg/kg, given 15 min before ethanol or ethanol as a 25% solution, w/v in normal saline, or sodium pentobarbitone, 60 mg/kg, were injected intraperitoneally.

As shown in Fig. 1, the time for the return of righting reflex after pentobarbitone injection was increased by propranolol. However the return of the righting reflex after ethanol was shortened by low doses of propranolol but increasing the dose caused the righting reflex time to rise, roughly paralleling the slope for the barbiturate curve.

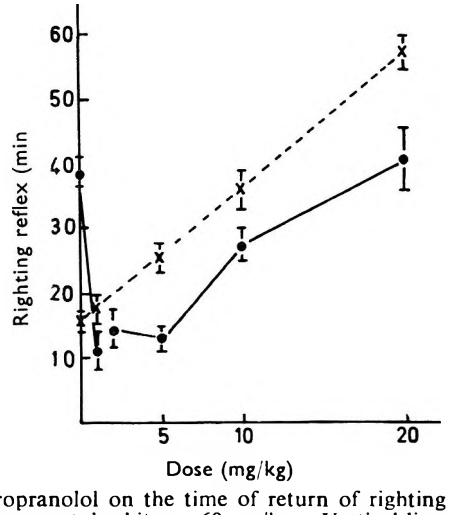

FIG. 1. The effect of propranolol on the time of return of righting reflexes after injection of —— ethanol, 4 g/kg or - - - pentobarbitone, 60 mg/kg. Vertical lines indicate s.e.

The finding that a β-adrenergic blocking agent can inhibit the central depressant actions of ethanol, but not pentobarbitone, suggests that perhaps brain noradrenaline modulates the depressive response to ethanol but not to pentobarbitone. A similar modulating effect of noradrenaline is suggested by the fact that reserpine inhibits the analgesic effect of morphine (Sigg, Caprio & Schneider, 1958) whereas it is enhanced by adrenaline. On the other hand, the respiratory depression produced by morphine was not inhibited by propranolol pretreatment (Smith & Hayashida, 1970).

The specificity of propranolol for ethanol depression was tested using several other alcohols. Methanol, isopropanol or propanol were injected intraperitoneally in doses of 6, 3 or 2 g/kg so as to produce loss of righting reflex for 27 to 54 min. Pretreatment with propranolol increased the depressant effect of methanol and propanol by 33 and 42% whereas propranolol shortened by 35% the righting reflex time for isopropanol. A control study using ethanol, 4 g/kg, showed that propranolol blocked the depressant effect of ethanol by 54%. In each instance the change in

righting reflex time, compared with control, was significant at P, 0·05. Evidently propranolol was more effective against the depression induced by 2 carbon alcohols.

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Insulin tolerance in thiamine-deficient rats

In studies on thiamine-deficiency, we reported that the tissue concentration of catecholamine was significantly elevated in thiamine-deficient rats (Iwata, Fujimoto & others, 1968) and that monoamine-oxidase is involved in accumulation of endogenously formed catecholamine in tissues in thiamine-deficiency (Iwata, Nishikawa & Fujimoto, 1969a). Further, we recognized that a sedative effect was rarely seen in deficient animals given reserpine, but that when present, sedation was possibly due, at least in part, to slow depletion of the elevated catecholamine level caused by thiamine-deficiency (Iwata, Watanabe & Nishikawa, 1969b). Wien (1936) found that rats deprived of all the vitamin B complex exhibited a greater hypoglycaemic reaction to insulin than animals on a normal diet. On the other hand, Burke & McIntyre (1938) showed that thiamine added to the diet of rats, increased their hypoglycaemic response to insulin.

On the basis of these results, we examined the differences in behaviour and changes in blood sugar in control and thiamine-deficient rats caused by toxic or lethal doses of insulin. Thiamine-deficient rats were obtained as described by Iwata & others (1969a).

When control animals, on diet supplemented with thiamine, and pair-fed animals, showing a loss of body weight similar to that of the thiamine-deficient group, received 100 i.u./kg insulin intraperitoneally, they generally showed reduction in spontaneous motor activity about 1·5 h later and then developed tremor followed by clonic convulsions. They invariably showed symptoms of severe collapse after about 2·5 h and died after about 3·2 h. However, in the thiamine-deficient group only a very slight decrease in spontaneous motor activity was observed and neither convulsions nor prostration were seen within 12 h after administration of insulin. Moreover, as shown in Table 1, no animals in the deficient group died. When thiamine-deficient animals had been injected with 4 mg/kg thiamine hydrochloride 5 h previously, they developed the same symptoms as the control and pair-fed rats after injection of insulin, and four of the five animals died within about 7 h after administration of insulin.

Insulin shock is thought to be due to the hypoglycaemic effect of the hormone, because patients usually recover from insulin coma on infusion of glucose. The effect on the blood sugar level of a large dose of the hormone (100 i.u./kg, i.p.) was examined in deficient, control and pair-fed animals to see whether deficient rats showed a hypoglycaemic response to insulin. As seen in the Fig. 1, after this dose of insulin, the blood sugar level of control animals decreased by about 50% after 1 h,

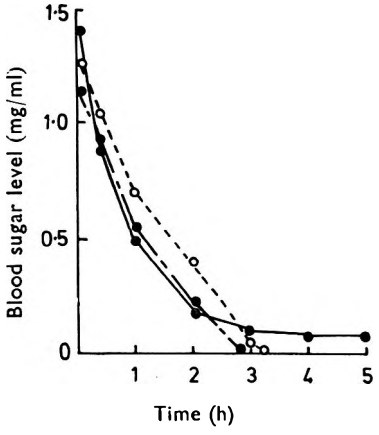

FIG. 1. Effect of insulin (100 i.u./kg, i.p.) on the blood sugar level of rats. Each point represents the mean of values of 3 to 5 animals. ●—● Thiamine deficient; ○--○ control; ●--● pair fed.

Table 1. *Effect of thiamine-deficiency on the lethal effect of insulin.* Insulin (100 i.u./kg) was injected intraperitoneally. Thiamine hydrochloride (4 mg/kg) was injected subcutaneously 5 h before insulin.

	No. of animals	Mortality (%)
Control	28	100
Thiamine-deficient	26	0
Pair-fed	8	100
Thiamine-deficient + thiamine	5	80

and by 70% after 2 h and was undetectable after 3·2 h. The blood sugar of thiamine-deficient animals decreased more rapidly than that of control animals for the first 2 h after insulin administration, but then decreased only slightly and was 0·07–0·08 mg/ml after 5 h.

This is the first report of the unexpected phenomenon that thiamine-deficient rats, unlike control and pair-fed animals show, no toxic reactions, such as convulsions and collapse, after a large dose of insulin. Based on our previous findings (Iwata & others, 1968) it seems possible that increased endogenous catecholamines in the brain or in other tissues caused by thiamine-deficiency may be responsible for this resistance to insulin toxicity. Furthermore, though the sustained low level of anthrone positive substance in the thiamine-deficient group after insulin is unknown, it seems possible that utilization of amino-acids or carbohydrates other than glucose in brain metabolism is enhanced in thiamine-deficiency.

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Acetylcholine release from cortical brain slices of rats injected with lithium

Waziri (1968) found that the addition of Li^+ (10–20 m-equiv/litre) to the fluid bathing Aplysia ganglion cells resulted in pre-synaptic electrical changes consistent with decreased acetylcholine release. Katz, Chase & Kopin (1968) reported decreased release of [^{3}H] 5-hydroxytryptamine or [^{3}H]noradrenaline after stimulation of brain slices from rats previously injected with lithium chloride (2·5 or 7·5 m-equiv/kg 48 and 24 h before) and also from non-injected rats when lithium chloride (1·2 or 2·4 m-equiv/litre) was added to the incubation medium. To ascertain directly the effect of lithium upon acetylcholine release from mammalian brain, we injected rats with lithium and measured the release of endogenous acetylcholine from electrically-stimulated cortical brain slices prepared from these animals and from controls injected with sodium chloride.

Male white Sprague-Dawley rats (150–200 mg) were injected with lithium chloride or sodium chloride (2·5 m-equiv/kg intraperitoneally) twice a day for four days. Three h after the last dose of lithium the rats were decapitated and the brains were removed. Cortical slices were rapidly prepared and mounted for electrical stimulation between quick-transfer electrodes in a water bath (Bowers, 1967). All slice experiments were made in Tyrode solution containing Li^+ 5·0 m-equiv/litre. We had previously determined that the addition of this quantity of lithium to Tyrode solution did not alter the electrically stimulated release of acetylcholine from cortical brain slices of rats which had not been previously injected with lithium. In most stimulation experiments slices from lithium-injected animals and sodium-injected animals were stimulated simultaneously. In some experiments incubated but unstimulated control slices were also added to this group. Acetylcholine was measured by bioassay using the Venus heart (Bowers, 1967). We found that the addition of lithium chloride to the bioassay bath (up to 10^{-4} g/ml) had no obvious effect upon the spontaneous beat of the LSD-stimulated clam heart nor did it affect the shape of the dose-response curves to standard acetylcholine solutions.

Under these conditions, there was no significant difference in the electrically-stimulated release of endogenous acetylcholine from slices prepared from sodium-injected [0·86 $\pm$ 0·07(17)] as compared to lithium-injected [0·92 $\pm$ 0·08 (20)] rats (doses as μg/g of brain, wet weight). Mean values for both these groups were significantly greater than values from control unstimulated slices [0·56 $\pm$ 0·05 (12)] ($P < 0{\cdot}01$). These results do not support the hypothesis that lithium decreases acetylcholine release in mammalian brain. On the other hand, they do not exclude the possibility that certain more physiological forms of acetylcholine release in brain might be influenced by lithium.

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Action of cobalt compounds on vascular permeability, mast cells and blood clotting in rats and rabbits

Recently Mariano, Maria de Lourdes & others (1969) described a cobalt-induced immediate increase in vascular permeability of the rat skin, while at the same time the morphological aspects of the mast cells were not altered within 80 min after intraperitoneal injection.

In our experiments with Wistar rats the degranulation of peritoneal and tissue mast cells began 1 h after intravenous injection of cobalt(II) glutamate (1 mg Co/kg) (Fiedler & Hahn von Dorsche, 1969). The selective uptake and enrichment of ^{58}Co by mast cells in rats were proved by autoradiography (Hahn von Dorsche & Fiedler, 1970). In spite of the degranulation of the mast cells neither thrombo-haemorrhagic phenomena [subcutaneous injection of adrenaline, 0·25 mg/kg, 30 min before and simultaneous with the intravenous injection of cobalt(II) glutamate] nor acute conditioned necrosis (Selye, Rohan & others, 1966) could be produced.

In rabbits, rats and guinea-pigs, injection of cobalt(II) salts (2–5 mg Co/kg) alters the conformation of fibrinogen (Fiedler, 1969; Krantz, Fiedler & Lober, 1970). The prothrombin and thrombin time were much prolonged and the plasma formed a jelly instead of a normal fibrin clot (Taube, Fiedler & Hartmann, 1967). Spontaneous bleeding was not observed after a single injection of cobalt. But after repeated injections of cobalt(II) glutamate or cobalt chloride (5 mg Co/kg) subcutaneously) in 10 h intervals the rabbits looked worse, refused food and showed diarrhoea. In such animals killed after 3–4 days we observed expanded bleeding regions in lung, pericardium and abdomen (especially in the liver), and suggillations in the subcutaneous tissue. Detailed histological experiments about the localization of the damage are in progress.

The application of cobalt(III) complexes did not disturb the blood clotting in rabbits. Otherwise the single injection of tris(ethylenediamine)cobalt(III) chloride and hexammino-cobalt(III) chloride (5 mg Co/kg subcutaneously) caused spontaneous bleeding in the abdomen. Similar experiences were reported from experiments in frogs (Oswald, 1922). The cause of the disturbed capillary permeability may be due to binding of cationic cobalt(III) complexes to anionic mucopolysaccharides of the connective tissue (Bychkov & Khazanova, 1965; Mathews, 1964).

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Inhibition by propranolol of ethanol-induced narcosis

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Acetylcholine release from cortical brain slices of rats injected with lithium

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Action of cobalt compounds on vascular permeability, mast cells and blood clotting in rats and rabbits