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Research Papers

An investigation of die wall friction during the compaction of powders

C. J. LEWIS† AND DAVID TRAIN*

Crystalline aspirin, hexamine, sucrose, and sodium chloride, and simple granulations of hexamine and of sucrose, have been compressed and the die reaction determined using a "moving-die" technique. The shear strength of the ejected compacts has been measured under zero load conditions, and when compressed to theoretical density, using a punch penetration test. Calculated values of die reaction based on friction theory have been compared with experimental measurements. Correlation between the two is best for aspirin and hexamine crystals when the compact approaches theoretical density and shear strength values for compacts of zero porosity are used in the calculations; for sodium chloride, correlation is best when shear strength values under zero load conditions are used.

EXPERIMENTAL results on the pressing of powders in cylindrical dies (Duwez & Zwell, 1949; Spencer, Gilmore & Wiley, 1950; Ballhausen, 1951; Sheinhartz, McCullough & Zambrow, 1954; Toor & Eagleton, 1956) conform to the exponential relationship: (see Lewis & Train, 1965)

$$\log_e F_a/F_b = 4\mu\eta L/D \quad .. \quad .. \quad .. \quad (1)$$

Basic frictional research has shown that the value of μ may be modified by the stress normal to the interface (Pascoe & Tabor, 1956) and by relative interfacial movement (Courtney-Pratt & Eisner, 1957).

In some instances of powder compaction (Ballhausen, 1951; Sheinhartz & others, 1954; Toor & Eagleton, 1956) there was uncontrolled movement because the bottom punch was required to move to actuate the force measuring device; in others (Duwez & Zwell, 1949; Spencer & others, 1950; Train, 1956; Train, 1957) movement at the bottom punch was negligible since the bottom punch was virtually fixed to the die. Spencer and his colleagues (1950) induced relative movement between the die and the material in order to measure the limiting coefficient of die wall friction, and a moving-die technique designed to standardise relative movement has been reported (Train, Carrington & Hersey, 1962).

A punch penetration test has been used to measure the shear strength of solid specimens of talc and graphite (Train & Hersey, 1960a) and other materials (Hersey, 1960) and it was shown (Hersey, 1960), using idealised systems, that the Bowden and Tabor theory of friction (Bowden & Tabor, 1954) could be applied to a compacting system.

Practical values for the shear properties of small crystals are difficult to obtain. The present work describes an investigation of the frictional

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behaviour of some crystalline materials and an attempt to correlate die wall friction with the shear strength of the compact.

MATERIALS AND APPARATUS

The apparatus used has been described elsewhere (Lewis & Train, 1965).

Crystalline samples of sodium chloride, hexamine, aspirin, and sucrose were sieved using British Standard sieves on an Inclyno machine for 15 min. 40–60 mesh sodium chloride and 30–40 mesh fractions of the other materials were used.

Simple granulations of sucrose and of hexamine were made by hand, using crystalline material which had been ball-milled until all passed a 100 mesh sieve, and distilled water as a binding agent. The sucrose granules were dried for 2 hr at 60°; the hexamine granules were dried at room temperature for 1 hr and then at 50–55° and 29 in Hg vacuum for 2 hr. The dried granulations were sieved and 30–40 mesh fractions used.

Experimental

5 g samples of the test materials were compressed in a die with 2·41 cm bore at pressures up to 5,000 kg/cm^2. The rate of application of pressure was constant at 638 kg/sec and the die was moved relative to the compact at 0·22 cm/sec. The compacts were ejected from the die, their weight and dimensions measured, and the shear strength determined immediately using a punch penetration test (Train & Hersey, 1960a); the shear force was applied at a constant rate of 166 kg/sec.

The applied force, the die reaction, F_d, and the ejection force, F_e, were recorded using a U.V. recorder (Lewis & Train, 1965); the change in length of compact with pressure was also measured.

To assess the effect of wall friction on the process of consolidation only, 2% magnesium stearate powder —100 mesh, was added to further quantities of test material. 5 g quantities of the lubricated materials were compressed in the die in an identical manner and the change in density of the compact with load was determined.

Three compacts of each unlubricated crystalline material were made of such a weight that their length at zero porosity was 0·5 cm; the compaction pressure used was 1,000 kg/cm^2. The compacts of hexamine, aspirin, and sodium chloride, were subjected to a compressive load in the shear strength apparatus so that the porosity was nil, and the shear strength was measured in the normal way. The pressure required to reduce the sucrose compacts to zero porosity (4,250 kg/cm^2) was beyond the capabilities of the apparatus, so the shear strength of sucrose compacts under load was measured at the maximum pressure available (3,000 kg/cm^2), at which pressure the porosity of the compacts was 3%.

Results and discussion

SHEAR STRENGTH OF COMPACTS

With the exception of sodium chloride, the shear strength of the compacts attained peak values within the range of compaction pressures

used (Fig. 1). For the sake of clarity results for sucrose granulation are given in Table 1.

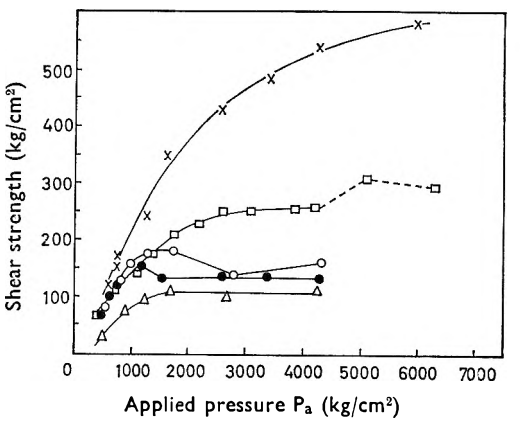

FIG. 1. Effect of applied pressure on shear strength of compact. × Sodium chloride. □ Sucrose. ○ Hexamine. ● Hexamine granulation. △ Aspirin.

TABLE 1. THE EFFECT OF APPLIED PRESSURE ON THE SHEAR STRENGTH OF COMPACTS OF SUCROSE GRANULATION

Applied pressure Pa kg/cm²	Shear strength kg/cm²
547	33
872	68
1,715	211
2,920	124

The decrease in shear strength observed at $P_a = 2{,}920$ kg/cm² was associated with the appearance of lamination lines in the compact. Compaction to greater pressures resulted in compacts which split into horizontal layers when ejected from the die and which expanded radially to such an extent that they could not be inserted into the bore of the shear strength apparatus.

The maximum shear strength of compacts prepared from sucrose, hexamine, and hexamine granulation also corresponds to the appearance of lamination lines, but aspirin compacts reach a maximum strength with no apparent fracture lines in the compact. The compaction pressures at which maximum shear strength is attained are given in Table 2.

The applied pressure for hexamine is that at which the compact approaches zero porosity under load (Fig. 3B) and a maximum density when ejected. Aspirin is fully consolidated under a load of approximately 1,000 kg/cm² and the pressure listed in Table 2 is that at which the porosity of the ejected compact becomes constant (Fig. 2B). Sucrose behaves in a similar manner to aspirin (Fig. 3A).

For these materials it would appear that the maximum shear strength under zero load conditions is associated not with the pressure at which

TABLE 2. APPLIED PRESSURE WHEN SHEAR STRENGTH BECOMES MAXIMAL

Material	Applied pressure, kg/cm²
Hexamine	1,500
Hexamine granulation ..	1,250
Aspirin	1,700
Sucrose	2,600
Sucrose granulation ..	1,715

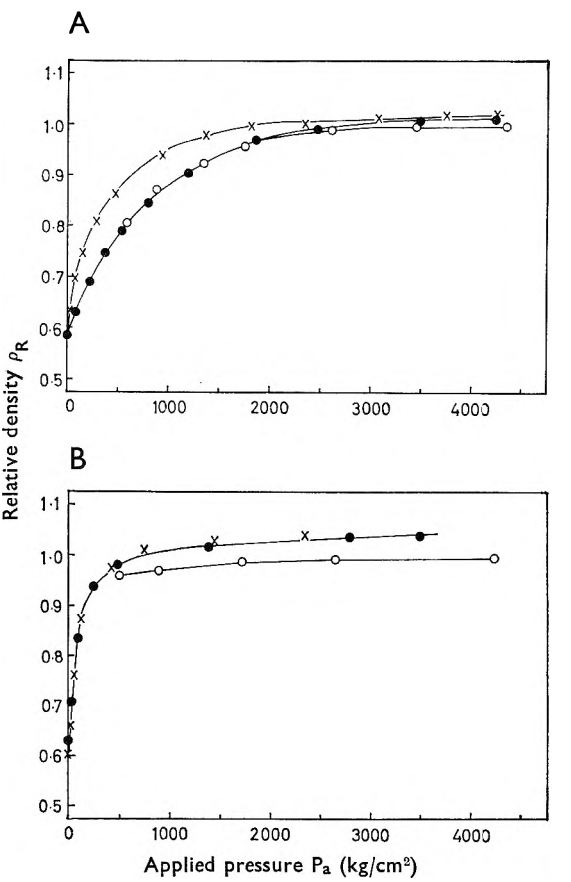

FIG. 2. Effect of applied pressure on density of compact. A. Sodium chloride. ● Unlubricated. × Lubricated. ○ Ejected and unlubricated. B. Aspirin. ● Lubricated. × Unlubricated. ○ Ejected and unlubricated.

consolidation is complete when compressed, but with the pressure necessary to produce minimum porosity in the ejected compact. Sodium chloride does not fit into this concept, for although the porosity of the ejected compacts becomes constant at applied pressures of 3,000 kg/cm²

and greater (Fig. 2A), the maximum value of shear strength is not achieved.

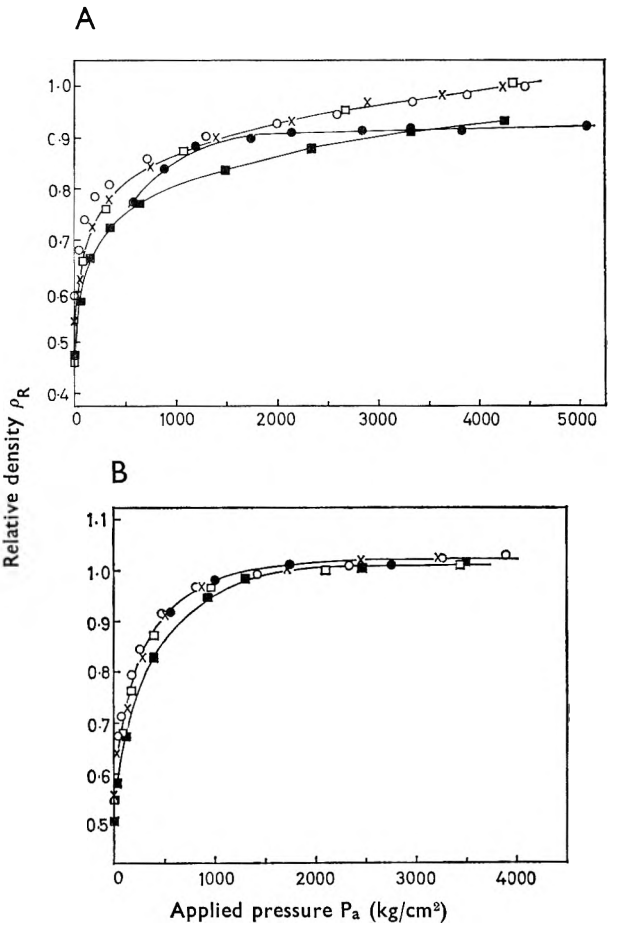

FIG. 3. Effect of applied pressure on density of compact. A. Sucrose. B. Hexamine. × Unlubricated. ○ Lubricated. ● Ejected and unlubricated. □ Granulation lubricated. ■ Granulation unlubricated.

TABLE 3. THE SHEAR STRENGTH OF COMPACTS UNDER AN APPLIED LOAD

Material	Shear strength kg/cm²	Porosity %
Hexamine ..	471	nil
Aspirin	297	nil
Sodium chloride ..	968	nil
Sucrose	1,112	3·0

Each result represents the mean of three determinations.

The shear strength of compacts under a compressive load is given in Table 3.

Shear strength values for compacts under load are much greater than

those for compacts under zero load, as might be expected from the results of Bridgman (1952) and Hersey (1960).

From the present results three values of shear strength of a compact may be chosen:

(1) Shear strength of a compact prepared at a given pressure and measured under zero load conditions, S_p;

(2) Maximum observed shear strength of a compact under zero load conditions (as in Fig. 1), S_m;

(3) Shear strength of a compact measured at zero porosity, S_o.

DIE REACTION

Hersey (1960) indicated that the friction theory of Bowden and Tabor was applicable to his compacting systems and that the die reaction,

$$F_d = S.A \qquad (2)$$

where S = shear strength of material being compressed;

A = true area of die wall—compact interface.

If the shear strength of the material remains constant, it would be expected that the die reaction would attain a maximum value when the porosity of a powder compact became zero.

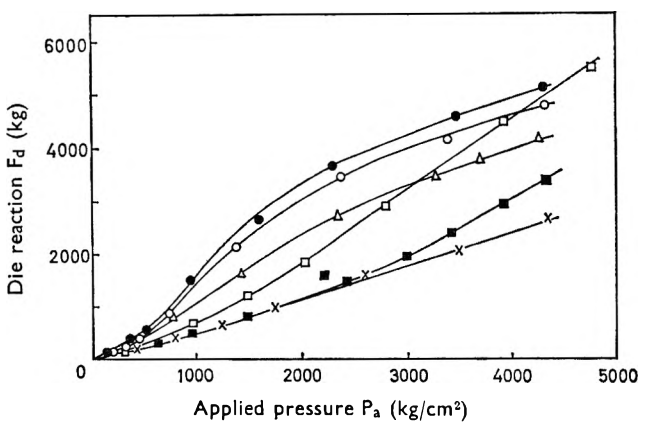

FIG. 4. Effect of applied pressure on die reaction. ○ Hexamine. ● Hexamine granulation. □ Sucrose. ■ Sucrose granulation. × Sodium chloride. △ Aspirin.

It was found that practical values of F_d did not attain a maximum at pressures up to 4,500 kg/cm² (Fig. 4), although the increase in F_d with pressure becomes less for hexamine, aspirin, and hexamine granulation, at applied pressures greater than 2,300 kg/cm².

The apparent area of compact—die wall contact, A_a, at a given compaction pressure can be calculated from observations of the length of compact. If it is assumed that the porosity is uniform throughout the compact, the true area of contact for compacts less than theoretical density is given by:

$$A = A_a.\rho_R \qquad (3)$$

$$\text{where } \rho_R = \text{relative density of compact} = \frac{\text{bulk density of compact}}{\text{density of solid}}$$

Values of F_d were calculated from equation (2) using experimental values of S_p and S_o, and are compared in Table 4 with the measured values for the four crystalline materials, over a range of applied pressures. At applied pressures greater than those necessary to produce maximum values of shear strength, F_d was calculated using S_m in place of S_p.

TABLE 4. COMPARISON OF EXPERIMENTAL RESULTS FOR F_d WITH CALCULATED VALUES

Material	Applied pressure Pa kg/cm²	Relative density under load ρ_R	Exptl. results F_d kg	Calculated F_d values	
				$A.S_p(S_m)$	$A.S_o$
Hexamine..	4,000	1·03	4,500	1,099	2,878
	3,500	1·03	4,200	1,099	2,878
	3,000	1·02	3,850	1,110	2,905
	2,000	1·02	2,950	1,115	2,920
	1,500	1·01	2,300	1,127	2,950
	1,000	0·98	1,400	986	2,905
	500	0·91	450	432	2,698
	250	0·82	200	155	2,431
Sodium chloride..	4,000	1·01	2,300	2,002	3,690
	3,000	1·00	1,800	1,772	3,725
	2,000	0·98	1,150	1,369	3,630
	1,500	0·94	800	1,085	3,500
	1,000	0·88	500	694	3,280
	500	0·78	250	270	2,907
	250	0·70	100	54	2,608
Aspirin ..	4,000	1·04	4,000	632	1,702
	3,000	1·04	3,200	634	1,713
	2,000	1·03	2,300	641	1,730
	1,500	1·02	1,700	627	1,739
	1,000	1·01	1,100	473	1,757
	500	0·98	500	176	1,720
	250	0·94	250	84	1,650
Sucrose ..	4,000	1·00	5,250	1,308	5,780
	3,000	0·97	3,850	1,269	5,610
	2,000	0·93	2,450	1,092	5,400
	1,500	0·90	1,750	870	5,230
	1,000	0·87	1,000	610	5,030
	500	0·81	400	325	4,700
	250	0·75	150	196	4,360

Consideration of values of $A.S_p$ (S_m) indicates that the discrepancy between calculated values and experimental measurements is greatest for aspirin, hexamine, and sucrose, at the higher compaction pressures. For sodium chloride and sucrose values of $A.S_o$ are larger than the measured values over the whole range of applied pressures used; for hexamine and aspirin the calculated values are larger than the practical results at low applied pressures, and smaller at high values of P_a. In the instance of aspirin, experimental and calculated values are comparable when $\rho_R = 1{\cdot}02$; the two values for hexamine are comparable when $\rho_R = 1{\cdot}02$ at an applied pressure of 2,000 kg/cm². Of the results listed in Table 4 the best overall relation between experimental measurements of F_d and calculated values is for sodium chloride when shear strength values at zero load (S_p) are used in the calculations.

The material being compacted undergoes maximum shear at the die wall, sufficient in the case of lead shot to cause the production of a compact

held together by the outside skin (Train & Hersey, 1960b). It is likely, therefore, that for the softer materials hexamine and aspirin, the true area of contact at the die wall at low pressures will be greater than that predicted from porosity observations, and would account for calculated values of F_d being less than the practical results. The higher practical values for F_d when P_a is large may be due to extrusion of the compacting material past the punch tip—of which there was some evidence—making the actual area of contact greater than the calculated value; or the shear strength of the material may be greater at these high applied pressures than the value measured at zero porosity.

To test this latter statement is beyond the capability of the apparatus available at present, but it is considered that the amount of extrusion of material that took place was insufficient to account for the discrepancy between practical and calculated results.

EJECTION FORCE

Figure 5 depicts the effect of applied pressure on ejection force. Of note is the much larger force needed to eject compacts of crystalline hexamine

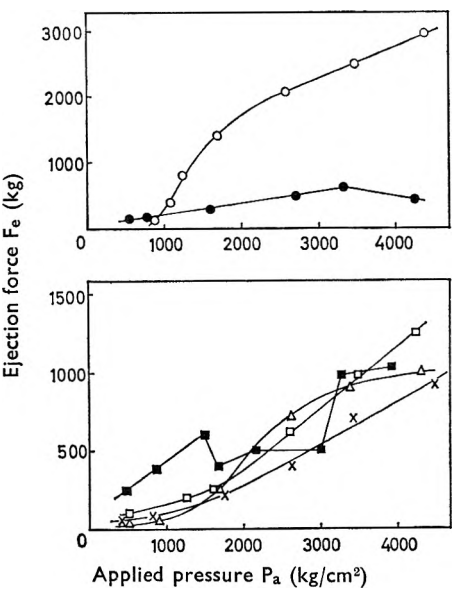

FIG. 5. Effect of applied pressure on ejection force. ○ Hexamine. ● Hexamine granulation. □ Sucrose. ■ Sucrose granulation. △ Aspirin. × Sodium chloride.

as compared to the granulation, since the latter would be expected to present a larger surface to the die wall at any one pressure than would the crystals because the granules were friable and the basic particle smaller.

Ejection forces for compacted sucrose granulation increase at first with increased compaction pressure but then decrease when $P_a =$

1,770 kg/cm^2 before again increasing. This decreased ejection force occurs at the point where lamination of the ejected compact becomes noticeable and where the compact undergoes appreciable expansion. It is possible that at applied pressures in the range 1,770–3,000 kg/cm^2 the compact is subject to axial recovery after removal of the load, causing the compact to laminate in the horizontal plane thus decreasing the residual radial stresses acting on the die wall. If radial stresses are diminished and all other conditions are the same, then a decreased ejection force would be needed.

Attempts to correlate numerical values of ejection force with the shear strength of the compact were completely unsuccessful, and visual observations of the behaviour of the compacted material during the experimental work indicated that the amount of elastic recovery of the compact after removal of the compacting pressure and before ejection, may well be as important as the shear strength of the friction junctions.

THE RELATIVE DENSITY OF THE COMPACT, ρ_R

Hexamine and aspirin attain zero porosity under load at comparatively low compaction pressures (Figs 2B, 3B), and the presence of lubricant has little influence on the degree of consolidation attained at any one compaction pressure. Sucrose and sodium chloride are more difficult to consolidate when unlubricated, and the addition of a lubricant causes these materials to attain a greater density at the lower pressures (Figs 2A, 3A).

The unlubricated granulations of hexamine and of sucrose are found to be more resistant to consolidation than the respective crystalline material, although the behaviour of lubricated samples is similar to that of the crystals. This behaviour has also been observed in tablets compressed with an instrumented tablet machine (Lewis, 1964). It is considered that this resistance is due to the presence of air trapped in the unlubricated granulation, whilst the lubricant facilitates particle sliding and possibly interferes with particle bonding in the early stages of compaction so allowing the air to escape.

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Effect of adrenalectomy on cotton pellet granuloma formation in the rat

A. ASHFORD* AND G. B. PENN

The development of granulomas over 3 days was inhibited in adrenalectomised rats when cotton pellets were implanted on the day of adrenalectomy and was potentiated when the pellets were implanted 7 days after adrenalectomy. The initial inhibitory effect of adrenalectomy was not observed when the granulomas were allowed to remain *in situ* for 7 days instead of 3 days and was completely reversed by treatment with corticosterone.

ALTHOUGH the actions of adrenal hormones on developing granulation tissue are widely known, there is some confusion about the effect of adrenalectomy upon healing wounds and granuloma formation. Whereas it has been reported that healing was retarded in adrenalectomised animals (Selye, 1947; Clayton & Prunty, 1951) and that the formation of granulomas around turpentine-induced abscesses in the rat was inhibited by adrenalectomy (Taubenhaus & Amromin, 1950), other workers have found that the amount of tissue formed around an implanted ivalon sponge (Pernokas, Edwards & Dunphy, 1957) or around a plastic ring (Jorgensen, 1962) in the rat was unaffected by adrenalectomy. Further reports record an increase in the amount of collagen produced in carrageenin-induced granulomas in adrenalectomised guinea-pigs (Robertson & Sanborn 1958) and an increase in the weight of carrageenin granulomas from adrenalectomised rats (Atkinson, Jenkins, Tomich & Woodlett, 1962) compared with intact controls.

Our observations show that effects of adrenalectomy ranging from inhibition to potentiation of granuloma formation can be elicited in the rat and are dependant upon the time that elapses between adrenalectomy and implantation of the irritant material.

Experimental

METHODS

Female Wistar rats, 130 and 180 g weight, were bilaterally adrenalectomised through a dorsal incision under ether anaesthesia. Controls were sham operated and non-operated animals; the latter were given ether only. The room temperature was 27° and the adrenalectomised animals received 0·9% saline to drink instead of tap water. Sterile cotton pellets each weighing between 6 and 10 mg (Johnson and Johnson) were implanted one in each groin under ether anaesthesia. Each batch of pellets was screened for irritancy since they varied in this property. The time of implantation in relation to adrenalectomy varied and is stated in the text (day 0 = implantation immmediately after adrenalectomy).

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The granulomas were removed 3 days after implantation of the pellet since they reach maximum weight at about this time (Penn & Ashford, 1963). In one experiment, granulomas were removed after 7 days. The granulomas from each rat were immediately weighed wet in pairs and in some instances after being dried overnight at 60°. The dry weights quoted are those of the dry tissue only.

DRUGS

Hydrocortisone acetate was administered as a saline suspension by a single i.p. injection immediately after implantation of the pellets. Controls received saline.

Corticosterone was dissolved in 5% ethanol. 8 i.p. injections each of 100 μg/kg, were given during the 3 day test period as follows: day 0, 1 injection immediately after implantation; day 1, 3 injections; day 2, 3 injections; day 3, 1 injection 2 hr before death. Controls received 5% ethanol (0·1 ml/100g i.p.).

Results

3 DAY OLD GRANULOMAS FROM COTTON PELLETS IMPLANTED AT 0, 3, 5 AND 7 DAYS AFTER ADRENALECTOMY

The weight of granulomas from adrenalectomised rats was dependant upon the time that elapsed between adrenalectomy and implantation of

TABLE 1. WEIGHT OF 3 DAY OLD GRANULOMAS IMPLANTED AT 0, 3, 5 AND 7 DAYS AFTER ADRENALECTOMY

Group	No. of rats	Day of implantation	Mean paired granuloma wt. mg ± s.e.			
			Wet	P	Dry	P
Non-operated	6	0	413·7 ± 18·1		—	
Sham-operated	10	0	367·4 ± 13·1	<0·001	—	
Adrenalectomised	10	0	290·4 ± 13·2		—	
Sham-operated	10	0	363·1 ± 19·8	NS	57·9 ± 4·9	<0·01*
Adrenalectomised	10	0	306·9 ± 23·2		34·9 ± 4·5	
Sham-operated	12	0	448·7 ± 16·4	<0·001	—	
Adrenalectomised	12	0	267·8 ± 8·0		—	
Non-operated	6	0	519·2 ± 42·6	<0·001	—	
Adrenalectomised	6	0	281·4 ± 17·4		—	
Non-operated	6	0	345·6 ± 19·1	<0·01	—	
Adrenalectomised	6	0	217·3 ± 24·2		—	
Non-operated	12	3	296·6 ± 17·7		42·8 ± 5·4	
Sham-operated	12	3	267·1 ± 19·1	NS	32·9 ± 13·9	NS
Adrenalectomised	12	3	306·6 ± 29·1		41·9 ± 6·8	
Sham-operated	12	5	281·8 ± 19·4	NS	40·5 ± 6·0	
Adrenalectomised	11	5	335·7 ± 26·2		49·2 ± 5·7	
Non-operated	24	7	341·4 ± 13·7		50·0 ± 3·9	
Sham-operated	24	7	375·7 ± 14·9	<0·001	57·4 ± 4·3	NS <0·01
Adrenalectomised	24	7	481·1 ± 24·6		68·8 ± 5·3	
Non-operated	10	7	477·5 ± 22·4		71·7 ± 5·3	
Sham-operated	10	7	493·1 ± 34·8	<0·05	83·9 ± 9·5	NS <0·01*
Adrenalectomised	10	7	600·9 ± 27·1		109·4 ± 8·4	

* Abstracted from Table 2, all rats having received control injections of 5% ethanol.
NS. Not significant P>0·05.

the pellets. Whereas the wet and dry weight was reduced by adrenalectomy when the pellets were implanted on day 0, there was statistically no significant difference in weight between adrenalectomised and intact groups when pellets were implanted on days 3 and 5. When the period between adrenalectomy and implantation was increased to 7 days the wet weight of granulomas from adrenalectomised rats was significantly greater than that of granulomas from sham-operated controls. However, the dry weight of the adrenalectomised group was not increased significantly ($P > 0.05$) compared with sham-operated controls but was significantly greater than non-operated controls ($P < 0.01$).

EFFECT OF CORTICOSTERONE ON 3 DAY OLD GRANULOMAS (Table 2)

Corticosterone treatment significantly increased the weight of granulomas from adrenalectomised rats implanted on day 0, the increase being

TABLE 2. EFFECT OF PARENTERAL INJECTIONS OF CORTICOSTERONE (8 × 100 μG/KG I.P.) ON THE WEIGHT OF 3 DAY OLD GRANULOMAS FROM COTTON PELLETS IMPLANTED AT 0 AND 7 DAYS AFTER ADRENALECTOMY

Group	No. rats	Day of implantation	Mean paired granuloma wt. mg ± s.e.			
			Wet	P	Dry	P
Sham-operated	10	0	363·1 ± 19·8	NS	57·9 ± 4·9	<0·05
Sham-operated + cortcosterone	10	0	313·6 ± 23·4		39·3 ± 5·9	
Adrenalectomised	10	0	306·9 ± 23·2	<0·02	34·9 ± 4·5	<0·01
Adrenalectomised + cortcosterone	10	0	422·8 ± 33·2		61·5 ± 7·3	
Non-operated	10	7	477·5 ± 22·4	<0·001	71·7 ± 5·3	<0·001
Non-operated + cortcosterone	10	7	255·9 ± 8·6		42·3 ± 3·4	
Sham-operated	10	7	493·1 ± 34·8	<0·001	83·9 ± 9·5	<0·001
Sham-operated + cortcosterone	10	7	249·3 ± 11·7		33·2 ± 3·1	
Adrenalectomised	10	7	600·9 ± 27·1	NS	109·4 ± 8·4	NS
Adrenalectomised + cortcosterone	10	7	517·9 ± 32·2		94·2 ± 8·9	

38% in the wet and 76% in the dry granuloma weight. The same dose of corticosterone had an anti-inflammatory action in sham-operated rats and reduced the dry granuloma weight by 32% ($P < 0.05$); the wet granuloma weight was also reduced (13%), although this reduction was not significant ($P > 0.05$).

When cotton pellets were implanted on day 7, corticosterone slightly reduced the weight of granulomas in the adrenalectomised group although the reduction was not significant ($P > 0.05$). There was a marked anti-inflammatory action in intact rats, the reductions being non-operated 46% wet and 41% dry, sham-operated 49% wet and 60% dry.

SEVEN DAY OLD GRANULOMAS FROM COTTON PELLETS IMPLANTED ON DAY 0

There was no difference between wet granuloma weights from sham-operated controls and adrenalectomised rats when the pellets remained *in situ* for a period of 7 days. For groups of 10 animals the figures were

337·9 ± 20·8 and 361·2 ± 26·6 for adrenalectomised and sham operated rats respectively.

ANTI-INFLAMMATORY ACTION OF HYDROCORTISONE ACETATE ON GRANULOMAS FROM PELLETS IMPLANTED ON DAY 0 (Fig. 1)

Relatively high doses of hydrocortisone exerted an anti-inflammatory action in adrenalectomised and in intact animals. The dose-response curves were parallel over the dose-range 25·0–50·0 mg/kg.

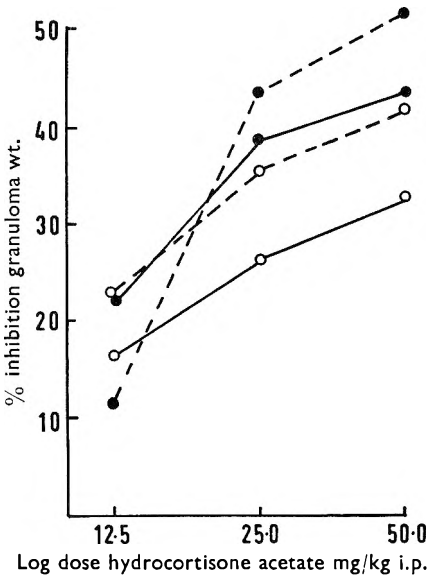

FIG. 1. The effect of hydrocortisone acetate on granulomas from cotton pellets implanted at the time of adrenalectomy and removed 3 days later. There were 8 rats per group. ●—● Non-operated wet weight. ○—○ Adrenalectomised wet weight. ● - - - ● Non-operated dry weight. ○ - - - ○ Adrenalectomised dry weight.

Discussion

Two clearly different effects of adrenalectomy have been shown—an inhibition of granuloma formation in acutely adrenalectomised rats and an increase in granuloma formation in rats implanted with cotton pellets 7 days after adrenalectomy. The transition stage was seen when implantation was on the 3rd and 5th day after adrenalectomy, the adrenalectomised rats responding in a similar manner at these times to intact controls. It is possible that much of the confusion in the literature about the effect of adrenalectomy on the formation of granulation tissue stems from this biphasic response to adrenalectomy.

The initial reduction in granuloma weight caused by adrenalectomy was reversed by small quantities of corticosterone which, however, had an anti-inflammatory action in intact rats. Presumably the different effect of corticosterone in adrenalectomised and intact rats was due to the small

dose used, all of it being needed in the former group of animals to correct the metabolic defect resulting from adrenalectomy and none being surplus for anti-inflammatory purposes. When a large excess of corticosteroid, in this case, hydrocortisone, was given to adrenalectomised rats an anti-inflammatory action was observed.

The inhibitory effect of adrenalectomy on pellets implanted on day 0 was not seen if these pellets were left *in situ* for 7 instead of 3 days.

A rebound increase in inflammatory response became evident after a delay of between 5 and 7 days following adrenalectomy. The delay suggests that some factor other than a simple deficiency of corticosteroids is involved. Long & Miles (1950) described a similar rebound effect in the response of intact immunised guinea-pigs to tuberculin after withdrawal of cortisone treatment. The increase in hypersensitivity in this instance was also preceded by a delay of several days and it was suggested that an increased secretion of thyroxine was responsible, this hormone having an action opposed to that of cortisone. Since thyroxine has been shown to promote the development of granulation tissue in hypophysectomised rats (Taubenhaus & Amromin, 1950) although not in intact animals (Taubenhaus, Taylor & Morton, 1952) it is possible that it may be implicated in the increased inflammatory response 7 days after adrenalectomy. On the other hand Spencer & West (1963), who studied the relationship between the thyroid and adrenal glands and their effect on histamine metabolism in the rat, concluded that there was no true balance between the glands, the anti-cortisone action of thyroxine being indirect and mediated via an alteration in the amount of corticosteroid available to the tissues.

It is likely that the stress of surgery in sham-operated control animals would result in an initial hypersecretion of corticosteroids which may affect the development of granulomas after implantation on day 0. In fact the weight of granulomas from sham controls after implantation on days 0 and 3 was less than those of non-operated controls although P exceeded 0·05 in both instances. Similarly, after implantation on day 7, sham-operated animals occupied an intermediate position between non-operated and adrenalectomised rats in respect of granuloma weight and showed a greater response to the anti-inflammatory effects of corticosterone than non-operated controls.

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Inhibition of rat brain glutamate decarboxylase activity by salicylate *in vitro*

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Salicylate inhibited rat brain glutamate decarboxylase activity *in vitro*. The mechanism of the inhibit on appeared to involve irreversible combination of the drug with the enzyme protein. Salicylate also decreased the incorporation of radioactivity from L-glutamic acid-^{14}C(U) into γ-aminobutyrate in preparations of rat brain, an effect consistent with an inhibition of glutamate decarboxylase activity by the drug.

SALICYLATE inhibits *Escherichia coli* glutamate decarboxylase *in vitro* (Gould, Huggins & Smith, 1963). In the present work the effects of salicylate on glutamate decarboxylase activity and the transfer of radio carbon from labelled glutamate to γ-aminobutyrate in rat brain preparations have been investigated.

Experimental

RAT BRAIN PREPARATION

Male Wistar rats, 200 to 300 g, maintained on M.R.C. cube diet no. 41, were killed by cervical fracture. The brain was removed, placed in the appropriate ice-cold buffer and weighed on a torsion balance. Two types of homogenate were prepared. The first, used for the determination of glutamate decarboxylase activity, was made in sufficient 0·05 M potassium phosphate buffer, pH 5·9, with a Dounce homogeniser, to give either a 20% w/v or a 40% w/v homogenate. The other, used in the radioactive experiments, was prepared in a similar manner using 0·01M potassium phosphate buffer, pH 7·4 to give a 33% w/v homogenate.

MEASUREMENT OF GLUTAMATE DECARBOXYLASE ACTIVITY

The method was based on that described by Tashain (1961). Assays were made in a standard Warburg apparatus at 37°. All solutions were prepared in 0·05M potassium phosphate buffer, pH 5·9. Two ml of the 20% w/v brain homogenate were placed in the main compartment of the Warburg flask and 1 ml of 0·3M L-glutamic acid containing 200 μg of pyridoxal phosphate, and the salicylate, when present, was placed in the side-arm. The manometers were flushed with nitrogen for 6 min, then equilibrated at 37° for a further 6 min. and the reaction started by tipping the glutamate solution from the side-arm. The CO_2 evolved was measured at 5 min intervals for 20 min and the values used to calculate the initial rate of the reaction. This was corrected for dissolved CO_2 by recalculation of the flask constants by the method described by Umbreit (1957).

Further experiments were made to define the mechanism of the inhibitory action of salicylate. Varying amounts of the 40% w/v brain homogenate were exposed to 150 mM salicylate for 30 min at 37° before

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the reaction was started by adding the glutamate solution to give a final volume of 3 ml.

RADIOACTIVE EXPERIMENTS

Portions (400 μl) of the 33% w/v brain homogenate were added to a solution containing 5 μc (0·5 μmole) of radioactive glutamate and the salicylate, when present, in 100 μl of 0·01M potassium phosphate buffer, pH 7·4. The L-glutamic acid-^{14}C(U) was obtained from the Radiochemical Centre, Amersham, Bucks. The mixtures were incubated at 37° with shaking and 100 μl samples removed at intervals of 5, 15, 30 and 60 min. After the addition of 400 μl of boiling ethanol, the samples were centrifuged and the radioactive substances present in the supernatants separated by two dimensional paper chromatography, visualised by radioautography and the ^{14}C measured by the techniques described by Smith & Moses (1960).

Results

The results in Table 1 show that salicylate inhibits glutamate decarboxylase activity in the rat brain preparation and that the degree of inhibition increased with the salicylate concentration.

TABLE 1. EFFECT OF SALICYLATE ON RAT BRAIN GLUTAMATE DECARBOXYLASE ACTIVITY

Final concentration of salicylate (mM)	Inhibition (%)
15	16
25	31
40	50
50	59
75	87
100	92
150	99

Each value represents the mean of six determinations. In the control experiments 26·0 μl of CO_2 were evolved during 20 min.

Table 2 shows the effect of exposing the rat brain preparation of glutamate decarboxylase to 150 mM salicylate before the reaction was started by the addition of the substrate. If the inhibitory effect of salicylate was reversible, then the observed degree of inhibition should have been

TABLE 2. EFFECT OF PRE-INCUBATING ENZYME PREPARATION WITH 150mM SALICYLATE

Final salicylate conc. in reaction mixture (mM)	Control rate (μl CO_2/20 min)	Theoretical inhibition (%)		Observed inhibition (%)
		Reversible	Irreversible	
25	12·8	31	99	99
50	26·0	59	99	99
100	50·3	92	99	99

determined by the final salicylate concentration in the reaction mixture. However, it was found that the inhibition persisted at the level induced by the salicylate concentration to which the enzyme preparation had been

exposed before the start of the reaction. The subsequent dilution of the 150 mM salicylate in the final reaction mixture therefore did not affect the degree of inhibition. This result indicated that salicylate causes an irreversible inhibition of the glutamate decarboxylase in the rat brain preparation.

The results in Table 3 show that the rat brain preparation incorporated radiocarbon from the labelled glutamate into γ-aminobutyrate and into glutamine showing that glutamate decarboxylase and glutamine synthetase activities were present. Salicylate caused a decreased formation of both labelled intermediates at all the times studied. Radioactivity also occurred

TABLE 3. EFFECTS OF 10mM SALICYLATE ON THE DISTRIBUTION OF RADIOACTIVITY FROM L-GLUTAMIC ACID-^{14}C(U) INTO THE SOLUBLE METABOLIC INTERMEDIATES OF THE RAT BRAIN PREPARATION

Results given as counts per min $\times 10^{-2}$ of ^{14}C.

Soluble intermediate	5 min		15 min		30 min		60 min	
	Control	Salicylate	Control	Salicylate	Control	Salicylate	Control	Salicylate
γ-Aminobutyrate	47·2	31·1	139·6	64·9	217·4	110·2	203·5	104·3
Glutamine ..	1·5	0·6	3·1	0	4·6	2·8	2·8	0·6
α-Oxoglutarate ..	55·0	69·9	52·6	38·1	18·7	33·2	15·6	22·5
Tricarboxylic cycle acids (succinate; fumarate: malate: citrate) ..	12·3	14·2	13·9	27·9	19·1	19·4	31·7	26·1
Aspartate ..	12·3	9·8	17·3	28·3	36·2	43·6	40·2	60·6

in α-oxoglutarate, various acids of the tricarboxylic acid cycle and in aspartate, showing that glutamate carbon had been transferred via α-oxoglutarate to the Krebs cycle. The aspartate was presumably formed by transamination of oxaloacetate. The salicylate did not cause either marked or consistent effects on the distribution of the isotope in these fractions except that the formation of labelled aspartate was increased at the later time intervals.

Discussion

Salicylate inhibits glutamate decarboxylase activity *in vitro* in the rat brain preparation used. The results of the radioactive experiments confirm this because the transfer of radioactivity from labelled glutamate to γ-aminobutyrate is markedly decreased in the presence of the drug. The radioactive work also provides evidence that salicylate interferes with the conversion of glutamate to glutamine, and Messer (1958) has shown that salicylate inhibits glutamine synthetase activity in mammalian brain.

The mechanism of the inhibitory action of salicylate on the rat brain glutamate decarboxylase is irreversible and is the same as that reported for the *E. coli* enzyme (Gould, Huggins & Smith, 1963). A possible mechanism is that salicylate combines with free amino-groups in the enzyme protein because it has been shown that the drug combines with ϵ-amino-groups in bovine albumin (Davison & Smith, 1961).

The finding that salicylate inhibits rat brain glutamate decarboxylase activity *in vitro* by an irreversible mechanism may have important implications *in vivo*. The drug interferes with other enzymes, but the inhibitions

are reversible. Thus many dehydrogenases are inhibited by salicylate but the mechanism involves competition with the pyridine nucleotide coenzymes (Hines & Smith, 1964) and may be reversed by the further addition of coenzyme even after the inhibition has become established (Smith, Bryant & Hines, 1964). Salicylate inhibits aminotransferase enzymes *in vitro* by competing with the amino- and oxo-acid substrates (Gould, 1964) and theoretically an *in vivo* inhibitory action of any particular salicylate concentration on these enzymes will disappear if the substrate concentrations increase. In addition, the effects of salicylate on these reversibly inhibited enzymes should progressively decrease as salicylate is removed from an *in vivo* system. This consideration does not apply to glutamate decarboxylase. Once the inhibitory effect of salicylate on the decarboxylase has become established it will not be altered by the subsequent removal of the drug, and presumably will persist until the enzyme protein is renewed. Thus it may cause a prolonged toxic effect in a patient poisoned with salicylate even when the drug has been removed from the body by renal excretion or haemodialysis.

If salicylate inhibits glutamate decarboxylase activity in the human brain, an expected result would be a decreased conversion of glutamate to γ-aminobutyrate. Altered ratios of the two amino-acids may occur in various regions of the central nervous system. There is experimental evidence (Way & Sutherland, 1963) that glutamate is a neuronal excitant whereas γ-aminobutyrate has an opposite action. A high glutamate: γ-aminobutyrate ratio resulting from an inhibition of glutamate decarboxylase activity, could cause a stimulation of the central nervous system and Roberts, Rothstein & Baxter (1958) have reported that a decrease in the brain glutamate decarboxylase activity produced convulsions in mice. Convulsions occur frequently in salicylate poisoning in man (Gross & Greenberg, 1948) and they may be mediated via an inhibitory action of the drug on glutamate decarboxylase in the brain.

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Modification of the hypoglycaemic response to tolbutamide and insulin by mebanazine—an inhibitor of monoamine oxidase

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Following reports of clinical changes in the carbohydrate metabolism of diabetic patients receiving mebanazine, possible interactions between mebanazine, adrenaline, tolbutamide and insulin have been studied in rats. Mebanazine had no activity on blood sugar levels and was found to have no effect on the metabolic actions of injected adrenaline. In acute experiments, mebanazine appeared to reduce the net hypoglycaemic action of tolbutamide but had no effect on the response to insulin. In chronic experiments, mebanazine pre-treatment led to a significant potentiation of the hypoglycaemic responses both to tolbutamide and insulin. The possible mechanisms of action and the clinical implications are discussed.

CHANGES in carbohydrate metabolism have been observed during the trial of a new antidepressive agent, mebanazine (Actomol, α-methylbenzylhydrazine). Clinical findings, in patients exhibiting both diabetic and psychiatric symptoms, included an improved glucose tolerance and lower fasting blood-sugar levels (Wickström & Pettersson, 1964). Most of these patients were receiving conventional antidiabetic therapy in addition to mebanazine, which is an inhibitor of monoamine oxidase. In certain cases the dosage of insulin or sulphonylurea had to be reduced to avoid hypoglycaemic crises. The opposing effects of catecholamines and insulin on blood-sugar are well established, so that the effects of mebanazine are unlikely to be due to inhibition of amine oxidase and prolongation of catecholamine action. However, in addition to their central antidepressive effects, the monoamine oxidase inhibitors appear to possess peripheral pharmacological actions which are not readily interpreted as a consequence of amine oxidase inhibition. For example, they have been shown to antagonise the release of catecholamines from adrenergic nerve endings (Brodie & Beaven, 1963) and to decrease the entry of noradrenaline into previously depleted storage sites (Davey, Farmer & Reinert, 1963). These pharmacological effects would be associated with a reduction in sympathetic activity. This potential inhibition of adrenergic function due to monoamine oxidase inhibitors might be particularly important in relation to tolbutamide therapy since this drug has been claimed to have direct catecholamine-releasing activity (Dulin, Morley & Nezamis, 1956; Bander, 1959). The present experiments were designed to determine whether or not the peripheral actions of mebanazine modified the hypoglycaemic responses to tolbutamide and to insulin.

Experimental

METHODS

The experiments were made using male rats (190–230 g) from the colony of specific pathogen-free albinos maintained at Alderley Park.

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Blood samples were obtained from the abdominal aorta after intraperitoneal pentobarbitone sodium anaesthesia.

Each treatment was given to groups of four animals except where otherwise indicated. Hypoglycaemic responses to tolbutamide (25, 50 or 100 mg/kg orally) were measured in rats which had been fasted overnight, primed with a subcutaneous injection of 100 mg of glucose (0·2 ml) and dosed orally with either saline or substance(s) under examination. Blood samples for analysis were taken 2 hr after dosing. The difference in blood-sugar between control and test groups was calculated as a percentage of the control values. Hypoglycaemic responses to insulin were measured in fed rats. The animals received 0·5 U soluble insulin subcutaneously and were bled 90 min later, food being withdrawn during this period.

Adreno-demedullated rats were maintained on 0·9% saline in place of drinking water for 4 weeks and used in experiments 6 to 8 weeks after operation.

The following determinations were made: blood-sugar (Hagedorn & Jensen, 1923); plasma free fatty acids (Dole, 1956); plasma tolbutamide (Toolan & Wagner, 1959); plasma corticosterone (Zenker & Bernstein, 1953 as modified by Barrett & Stockham, 1963); heart rates were recorded in pentobarbitone anaesthetised rats during intravenous adrenaline infusion by means of a cardiotachometer triggered by the QRS complex of the electrocardiogram.

Results

EFFECTS OF MEBANAZINE ALONE, AND ON THE RESPONSES TO INJECTED ADRENALINE

The effects of monoamine oxidase inhibitors on carbohydrate metabolism of rats and on the metabolic action of injected catecholamines do not appear to have been described in the literature. The fasting blood-sugar level has been determined in rats after 12 daily oral doses of mebanazine ranging from 2·0 to 30 mg/kg, there being 18 hr between the last dose and testing. No significant changes were found under these conditions nor 2 hr after a single dose of 120 mg/kg (Table 1). Other animals either

TABLE 1. EFFECT OF MEBANAZINE ON THE BLOOD-SUGAR LEVEL COMPARED WITH THAT OF TOLBUTAMIDE

Treatment		Time (hr) between last dose and sampling	Blood sugar level (mg/100 ml)
Drug	Dose		
Saline	0·5 ml/100 g	2	79 ± 3·0 (4)
Tolbutamide	100 mg/kg	2	43 ± 1·0 (4)
Mebanazine	120 mg/kg	2	80 ± 2·5 (4)
Mebanazine	60 mg/kg	18	90 ± 8·5 (4)
Saline	0·5 ml/100 g	2	
Mebanazine daily for 12 days	2·0 mg/kg	18	82 ± 3·0 (4)
	7·5 mg/kg	18	81 ± 5·5 (4)
	30·0 mg/kg	18	83 ± 2·0 (4)

pre-treated with a single dose of mebanazine (120 mg/kg) or else chronically treated for 12 days (30 mg/kg) were given intravenous infusions of

adrenaline. In neither group were the adrenaline-induced changes in blood-sugar, plasma free fatty acids or heart rate different from those observed in untreated control animals (Table 2). The results are in accord with those of Vanov (1962) who found that the pressor effects of injected adrenaline were not potentiated by monoamine oxidase inhibitors.

TABLE 2. THE EFFECT OF ACUTE AND CHRONIC PRE-TREATMENT WITH MEBANAZINE ON THE RESPONSE OF THE BLOOD-SUGAR, PLASMA FREE FATTY-ACIDS AND THE HEART RATE TO AN INTRAVENOUS INFUSION OF ADRENALINE (2 μg/kg/min) FOR 15 MIN. Each value represents the mean of observations in four rats with the standard errors of the means.

	Plasma free fatty acid concentration (μ-equiv./l)			Blood-sugar level (mg/100 ml.)			Heart rate (beats/min)		
Treatment	Controls	Infused	Difference	Controls	Infused	Difference	Initial	Final	Change
Controls—bled 15 min after pentobarbitone anaesthesia. Infused—bled 15 min after adrenaline infusion	353 ±33	630 ±42	+277	113 ±3·3	183 ±3·3	+70	409 ±18	514 ±11	+105
All animals received mebanazine (120 mg/kg) 150 min before proceeding as above	373 ±25	588 ±33	+213	100 ±5·2	175 ±11.0	+74	305 ±5	404 ±14	+99
All animals received mebanazine (30 mg/kg) daily for 12 days before proceeding as above	364 ±21	609 ±29	+245	107 ±4·9	179 ±4·8	+72	401 ±21	517 ±16	+116

The lack of effect of mebanazine on the blood-sugar level or on the responses to adrenaline is consistent with the suggestion of Axelrod (1960) that monoamine oxidase plays little part in the termination of the actions of catecholamines outside the central nervous system.

EFFECTS OF TOLBUTAMIDE OR INSULIN, ALONE

The hypoglycaemic effects of tolbutamide and insulin in the rat are well known. For control purposes, in each experiment standard doses of either tolbutamide (50 and 100 mg/kg) or insulin (0·5 U/100 g) were always given. In 20 experiments, the mean fall in blood-sugar to the lower dose of tolbutamide was 28 and 40% at the higher dose. Six weeks after adreno-demedullation, the hypoglycaemic response to tolbutamide was markedly enhanced. At a dose level of 100 mg/kg, demedullated animals became comatose within 1 hr of dosing and falls of up to 80% in blood-sugar were recorded. The mean fall in blood-sugar of intact rats given insulin was 40% in two experiments.

ACUTE EFFECTS OF MEMBANAZINE ON THE RESPONSE TO TOLBUTAMIDE AND INSULIN

Possible interactions between mebanazine and hypoglycaemic agents and the role of the sympathetic nervous system in the regulation of blood-sugar levels were therefore examined. When tolbutamide and mebanazine

were given together, the fall in blood-sugar was significantly smaller than that observed when tolbutamide was given alone (Fig. 1). There was also a reduction in the slope of the dose-response curve, for those animals receiving both drugs. The effect was not solely due to adreno-medullary stimulation as the antagonism was also found in demedullated rats (Fig. 2). Indeed, the effect was more dramatic since simultaneous administration of mebanazine prevented the blood-sugar dropping to fatal levels after tolbutamide. These animals did not become comatose and showed no observable signs of distress.

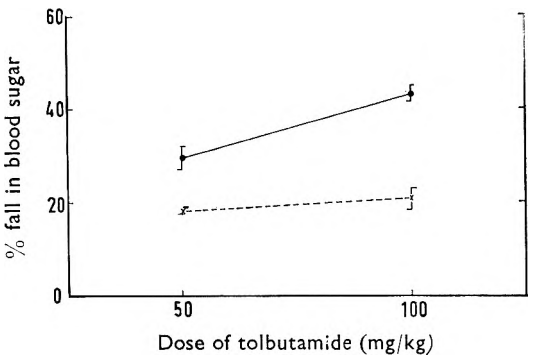

FIG. 1. The effect of tolbutamide on blood-sugar when given alone (O——O) or simultaneously with mebanazine, 120 mg/kg (X - - - X).

On the other hand, the degree of hypoglycaemia produced by insulin was the same in both control and mebanazine-treated rats, 2 hr after a single dose of the inhibitor (Table 3).

TABLE 3. THE EFFECT OF INSULIN ON BLOOD-SUGAR IN CONTROL RATS AND ANIMALS PRE-TREATED WITH A SINGLE DOSE OF MEBANAZINE 30 MIN BEFORE INSULIN. Blood samples were taken 90 min after insulin (i.e. 2 hr after mebanazine)

Dose of insulin	Dose of mebanazine	Blood-sugar level (mg/100 ml)	Fall in blood-sugar %
0	0	107 ± 9·5 (4)	—
0·5U/100 g	0	62 ± 5·2 (4)	42
0·5U/100 g	120 mg/kg	62 ± 1·5 (4)	42

It was likely that the antagonism between mebanazine and tolbutamide, under these conditions, resulted from the potentiation or unmasking of some property of tolbutamide rather than from an enhanced adrenergic response to hypoglycaemia *per se*. Evidence concerning the catecholamine-releasing potential of tolbutamide has been cited earlier (Dulin & others, 1955; Bander, 1959). Further, it is known that the pharmacological actions of substances which act by releasing noradrenaline, e.g., tyramine (Burn & Rand, 1958) are potentiated by monoamine oxidase inhibitors (Blackwell & Marley, 1964).

EFFECT OF CHRONIC MEBANAZINE TREATMENT ON THE HYPOGLYCAEMIC RESPONSES TO TOLBUTAMIDE AND INSULIN

The clinical improvements in diabetes mellitus were noted only after several weeks of treatment with mebanazine. To study the effects of

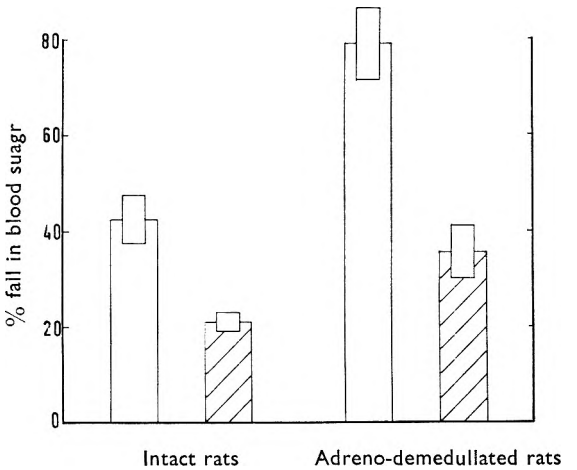

FIG. 2. The antagonistic effect of mebanazine, 120 mg/kg, on the hypoglycaemic response to tolbutamide, 100 mg/kg, in intact and adreno-demedullated rats. Hatched columns, both drugs. Open columns, tolbutamide alone.

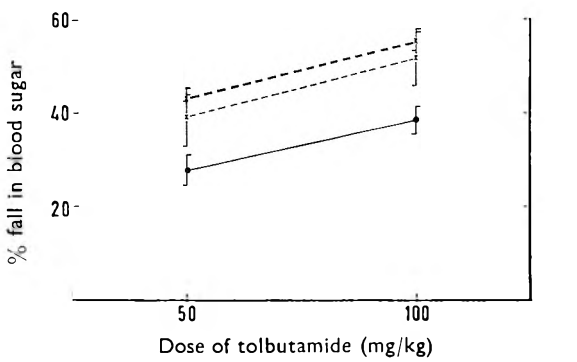

FIG. 3. The effect of tolbutamide on blood-sugar in control rats and 18 hr after the last of 21 consecutive daily doses of mebanazine. ●——● tolbutamide alone. X - - - X tolbutamide and mebanazine 15 mg/kg/day. X · · · X tolbutamide and mebanazine, 30 mg/kg/day.

chronic mebanazine treatment, rats were dosed orally for 3 weeks. After an overnight fast, without further mebanazine, the hypoglycaemic response to tolbutamide was assessed. The chronically-treated animals showed a significantly greater fall in blood-sugar after tolbutamide than that found in rats which had been dosed with saline for 3 weeks. There was no significant difference between the potentiation at 15 or 30 mg/kg of

mebanazine (Fig. 3). Similar potentiation occurred after 12 days of treatment at 7·5 to 30 mg/kg daily. Although the potentiation increased with dosage, the results at 7·5 and 30 mg were not statistically significant from one another (Table 4).

TABLE 4. THE EFFECT OF A SINGLE DOSE OF TOLBUTAMIDE ON BLOOD SUGAR, 13 HR after the last of twelve consecutive daily doses of mebanazine

Daily dose of mebanazine (mg/kg)	Dose of tolbutamide (mg/kg)	Fall in blood-sugar % (mean ± s.e.)	P value
0	100	37·7 ± 1·2 (4)	—
2·0	100	38·8 ± 2·7 (4)	>0·05
7·5	100	48·4 ± 4·0 (4)	<0·05
15·0	100	51·9 ± 5·7 (4)	<0·05
30·0	100	53·0 ± 2·4 (4)	<0·01

If the dose of mebanazine was increased to 60 mg/kg, given 13 hr before tolbutamide, there was as great a potentiation of hypoglycaemia as that seen after prolonged dosing at lower levels.

Similar results were obtained in groups of 9 rats chronically treated with mebanazine after receiving insulin in place of tolbutamide. Although only one dose level of insulin, 0·5 U/kg, was used the increase in hypoglycaemic effect was statistically significant after 12 days of treatment with mebanazine (15 mg/kg). The control value was 38 ± 4·9; treated value 51·0 ± 2·3: $P < 0·05$.

The results are compatible with the hypothesis that mebanazine produces partial inhibition of catecholamine release which normally occurs as a consequence of a fall in blood-sugar.

EFFECT OF CHRONIC DOSAGE OF MEBANAZINE ON GLUCOSE TOLERANCE

In an attempt to relate the present findings to the clinical observations, oral glucose tolerance curves were constructed for both control and chronically treated rats (Fig. 4). In both groups, the fasting blood-sugar level was the same and there was little difference in the 10 min peak value. However, the rate of fall in blood glucose was greater in the mebanazine-treated group, although only at the 20 min time interval was the difference between the two curves statistically significant ($P < 0·05$). Attempts to follow changes in intravenous glucose tolerance in rats were not successful owing to the rapidity with which blood-glucose falls after an intravenous injection of glucose.

CHRONIC EFFECTS OF MEBANAZINE ON HEPATIC AND ADRENOCORTICAL FUNCTION

Exaggerated hypoglycaemic responses have been reported after liver damage (Dall & Melrose, 1964). Hydrazines in general are potentially hepatotoxic and it is possible that mebanazine (which is, of course, a hydrazine) treatment might have affected the rate of hepatic inactivation of tolbutamide. Blood levels of tolbutamide 2 hr after dosing were not significantly different in either control or mebanazine-treated rats (Table 5).

In another experiment, the rate at which the blood tolbutamide level fell after a single 100 mg/kg dose was similar both in control rats and in animals which had received 30 mg/kg mebanazine daily for 3 weeks.

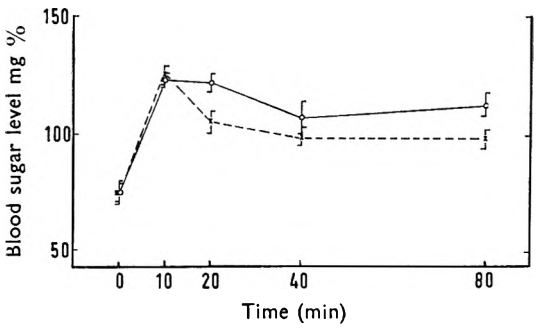

FIG. 4. The blood-sugar level in control and chronically mebanazine treated rats following an oral dose of glucose (10 g/kg). The animals were fasted overnight. O——O control rats. X - - - X mebanazine, 15 mg/kg/day.

TABLE 5. THE EFFECT OF CHRONIC TREATMENT WITH MEBANAZINE ON THE BLOOD LEVELS OF TOLBUTAMIDE, 2 HR AFTER ORAL ADMINISTRATION

Daily dose of mebanazine (mg/kg for 12 days)	Blood tolbutamide level (μg/ml)	
	After 50 mg/kg	After 100 mg/kg
0	282 ± 29 (4)	477 ± 38 (4)
2·0	230 ± 51 (4)	432 ± 51 (4)
7·5	328 ± 17 (3)	481 ± 31 (5)
30·0	281 ± 45 (3)	453 ± 46 (5)

The same mebanazine regimen produced a slight adrenal hypertrophy but no change in the resting plasma corticosterone concentration (controls 4·1 ± 1·3 μg/100 ml). The plasma corticosterone response to ether anaesthesia was not reduced by mebanazine treatment (controls 33·8 ± 4·7 μg/100 ml; mebanazine 46·6 ± 9·5 μg/100 ml).

Discussion

The results of these experiments in the rat afford a basis for the interpretation of the clinical observations in diabetic patients receiving mebanazine. Various mechanisms have been proposed to explain the orthostatic hypotension found as a side-effect of therapy with monoamine oxidase inhibitors. Apart from bretylium-like effects (Gessa, Cuenca & Costa, 1963), monoamine oxidase inhibitors with a hydrazine structure have been found to inhibit dopa decarboxylase and dopamine α-hydroxylase, both of which are important in the biosynthesis of catecholamines (Gey, Pletscher & Burkard, 1963). Preliminary studies suggest that mebanazine also possesses such properties. These pharmacological actions would serve to decrease both the stores and release of adrenergic transmitter resulting in reduced sympathetic activity. It is possible that the same

mechanisms are involved both in orthostatic hypotension and potentiation of hypoglycaemic agents. Co-existing hypotension and hypoglycaemia in a previously stable diabetic patient after mebanazine treatment was described recently (Cooper & Keddie, 1964).

Decreasing fasting blood-sugar levels and improved glucose tolerance in non-diabetic psychiatric patients was first noticed with iproniazid (Weiss, Weiss & Weiss, 1959). The clinical studies of Wickström & Pettersson (1964) together with the present findings, suggest that a certain kind of tolbutamide- and insulin-insensitive diabetic patient may not only show an improved response with mebanazine but that in certain cases the conventional antidiabetic therapy may be withdrawn. Mebanazine would appear to possess an insulin-sparing effect rather than a direct insulin-like hypoglycaemic action. Perhaps, therefore, certain patients diagnosed and treated for diabetes mellitus may in fact have no primary insulin deficiency, but rather, an oversensitive adrenergic hyperglycaemic mechanism which responds to a high critical blood-sugar level. If this is true, then hypoglycaemic agents would make the situation worse rather than better by stimulating further increases in adrenaline output which in turn would raise the blood-sugar level even higher. In the presence of a normal renal threshold to glucose, hyperglycaemia and glycosuria would persist in spite of rational antidiabetic treatment and only be antagonised by some form of adrenergic blockade.

A further implication of these experimental findings is that exaggerated hypoglycaemic responses may occur in patients receiving both antidiabetic therapy and mebanazine. It remains to be seen whether or not this applies to other monoamine oxidase inhibitors and adrenergic neurone blocking agents. Potentiation of insulin hypoglycaemia by ganglion blocking drugs has been reported in man, dog and rabbit (Laurence & Stacey, 1951; Schachter, 1951). Moreover, if the tolbutamide diagnostic test for either diabetes (Boshell, Wilensky, Wayland & Carr, 1963) or insulinoma (Fajans, Schneider, Scheingart & Conn, 1961) is conducted in persons also receiving drugs which interfere with adrenergic transmission, a false positive diagnosis is possible. Recently, there has been a report of erroneous diagnosis of insulinoma based on the intravenous tolbutamide test some years after sympathectomy (Cohn, Perlmutter, Silverstein & Numeroff, 1964).

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Cytological aspects of the mode of action of chlorhexidine diacetate

W. B. HUGO AND A. R. LONGWORTH

Electron microscopic examination of ultra-thin sections of *Escherichia coli* cells treated with various concentrations of chlorhexidine diacetate reveal two effects. In low drug concentrations many cells lose electron dense material leaving behind empty shells, in higher concentrations the appearance of the cytoplasm is significantly affected. These results are discussed in the light of biochemical findings under identical conditions.

THE adsorption of chlorhexidine by *Escherichia coli* cells and the resulting leakage of cell constituents and turbidity changes have been described by Hugo & Longworth (1964a). The effect of chlorhexidine on osmotically sensitive forms has also been described (Hugo & Longworth, 1964b). This work suggests that chlorhexidine exerts its bactericidal action by adsorption onto the cell surface and a reaction with the permeability barriers of the cell. Further evidence for the mode of action of chlorhexidine was sought by examining ultra-thin sections of *E. coli* cells, treated with chlorhexidine, by electron microscopy.

Experimental

Materials. The organism used in the work was *E. coli* (formerly NCTC 5934).

Culture media and conditions were as previously described (Hugo & Longworth, 1964a). Chlorhexidine diacetate (Hibitane) was a commercial sample (Imperial Chemical Industries Ltd.).

Araldite resin for embedding specimens was prepared from Araldite 502 CY212. 27 ml, Hardener HY 964. 23 ml, Accelerator DY 064, 2% (Ciba ARL Ltd.) as described by Glauert (1961).

Methods. Cells were harvested, adjusted nephelometrically and suspended at a final concentration of 1·2 mg dry wt cells/ml in 0·013M phosphate buffer pH 7·3 containing various concentrations of chlorhexidine at 20°. After each desired time interval for exposure of the bacteria to chlorhexidine had elapsed, fixation was according to the method of Kellenberger, Ryter & Sechaud (1958).

The samples were dehydrated, embedded in araldite resin and polymerised by the method of Luft (1961).

After sectioning with a diamond knife on a Cambridge Huxley ultramicrotome the sections were collected on copper grids stained for 1 hr in a saturated ethanolic solution of uranyl acetate (Watson, 1958) washed briefly in water and examined in an A.E.I. EM6 electron microscope.

The criteria used for making the choice of cells to record on film were those described by Chapman (1962): (1) Cells recorded are representatives of a definite majority of the cell population. (2) As nearly sagittal or transverse sections as possible are recorded.

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Results

Figs 1 and 2 show the appearance of typical *E. coli* cells after 1 and 6 hr suspension in phosphate buffer. Fig. 3 shows cells after 6 hr treatment with 20 μg/ml chlorhexidine. Little, if any, change is observable.

Fig. 4 shows the effect of 6 hr treatment with 90 μg/ml chlorhexidine. Many cells have lost all their electron dense material leaving empty shells believed to be the cell wall. Fig. 5 shows cells after 6 hr treatment with 200 μg/ml chlorhexidine. At this concentration empty cells are not seen but the cytoplasm presents a different appearance from that in the untreated controls.

The appearance of protuberances or adhesions on the cell surface may also be noted. Fig. 6 shows cells treated with 500 μg/ml chlorhexidine

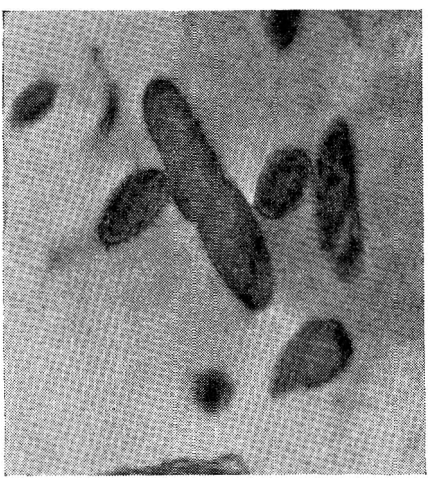

FIG. 1. *E. coli* cells suspended in buffer for 1 hr (× 16,250).

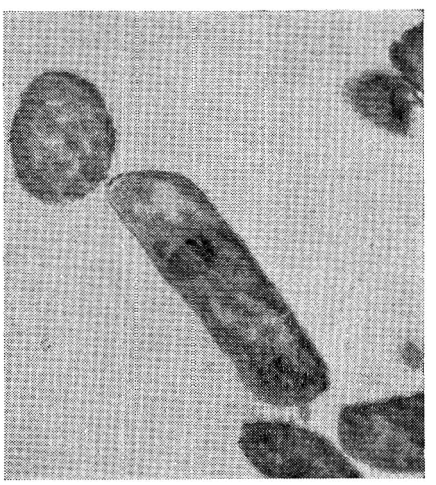

FIG. 2. *E. coli* cells suspended in buffer for 6 hr (× 20,000).

for 6 hr. Again, empty cells are not seen and the cytoplasm appears very granular. The protuberances or adhesions on the cell surface are larger and more numerous.

Further series of photographs taken after 1 and 3 hr contact with the same concentrations of drug as used above produced similar results and it would appear that of the two parameters, time and drug concentration, the second is more important with respect to chlorhexidine induced cytological damage.

Whilst it is accepted that in any population of cells the reaction to a stress will be variable it appears that three distinct effects of the drug depending on concentration may be differentiated. (1) Low concentrations, around 20 μg/ml, cause little or no observable cytological damage. (2) At concentrations around 90 μg/ml the drug causes cytological damage and loss of cell constituents. (3) At high concentrations, 200 and 500

μg/ml, cells do not appear to lose their cytoplasmic constituents. The cytoplasm, however, appears markedly different from that in the controls

FIG. 3. *E. coli* cells after 6 hr in buffer containing chlorhexidine, 20 μg/ml (× 16,250).

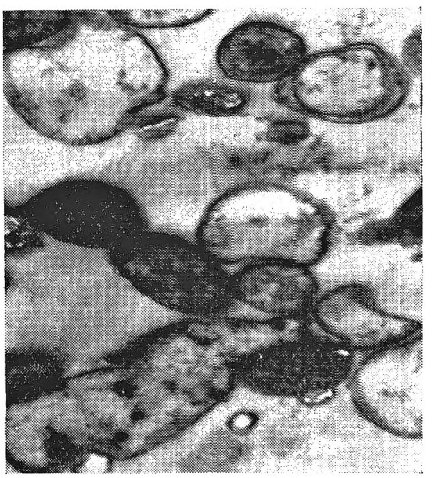

FIG. 4. *E. coli* cells after 6 hr in buffer containing chlorhexidine, 90 μg/ml (× 20,000).

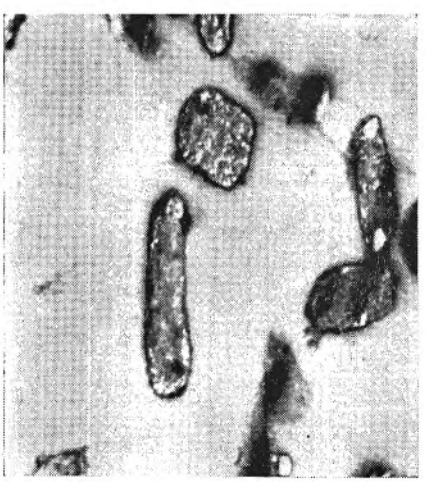

FIG. 5. *E. coli* cells after 6 hr in buffer containing chlorhexidine, 200 μg/ml (× 16,250).

FIG. 6. *E. coli* cells after 6 hr in buffer containing chlorhexidine, 500 μg/ml (× 20,000).

in presenting a granular appearance. The appearance of swellings on the cell surface at these concentrations is not fully understood but could be due to cellular extrusion or to the accumulation of drug aggregates on the cell surface.

Discussion

Hugo & Longworth (1964a) showed that treatment of *E. coli* cells with chlorhexidine causes a leakage of cell constituents into the suspending medium, indicating damage to the permeability barriers of the cell and that certain concentrations corresponding to a particular drug adsorption level cause maximal leakage and changes in turbidity. Higher concentrations of drug seemed to cause a low initial level of leakage which was not followed by a secondary leakage even though the drug, as would be expected, was more rapidly bactericidal, as estimated by extinction data, at high concentrations.

The results of the present communication show that, at the concentration of chlorhexidine which causes maximal leakage, cytological damage in the form of cell rupture leaving empty ghosts of cells is evident, whilst high concentrations seem to cause a change in the appearance of the cytoplasm without causing loss of cytoplasmic constituents. Newton (1953), using polymyxin E, which shows a diphasic leakage effect similar to chlorhexidine, also noted that shadowed preparations of *Pseudomonas aeruginosa* lose their "electron-dense" material when treated with a concentration of polymixin which causes maximal leakage but retain all electron dense-material when treated with high concentrations, whilst showing marked surface damage also in the form of surface swelling.

Chapman (1963), using colomycin which may be of an identical structure to polymyxin E (Wilkinson, 1963; Hugo & Stretton, 1963), showed that at a constant dose level of 1000 μg/ml, which is far in excess of the concentration causing maximum leakage (Hugo and Stretton, unpublished observations), the cytological effects can be divided into three types which, he suggests, result from an altered intracellular ionic milieu in turn due to a primary effect of the antibiotic agent upon the plasma membrane, the site of selective permeability.

If it is accepted that the cytoplasmic membrane in bacterial cells is responsible both for osmotic regulation and enzymic co-ordination of the cell then it is unnecessary to invoke two separate modes of action, one for low and the other for high concentrations of the drug. That a reaction occurs with the permeability barriers of the cell is evident both from biochemical and cytological studies, and the manifestations of cell death so caused, in terms of release of cell constituents, depends upon the level of drug adsorption. The gross cytoplasmic disorganisation occurring in high concentrations of the drug could be caused by disruption of the enzymic co-ordinating function of the cytoplasmic membrane which could in turn be caused by a disruption of the structure of the membrane, although, in contrast to low concentrations, this damage is not revealed by the loss of cytoplasmic constituents.

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An apparatus for the investigation of die wall friction during compaction

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A "moving-die" apparatus is described for use in the investigation of die wall friction during the compaction of powders. The powder can be compressed at constant rates varying from 344–860 kg/sec; the die can be moved at a constant rate at values of 0·1–0·25 cm/sec. The apparatus can also be used to apply the force necessary to cause shear in a punch penetration test for the measurement of shear strength of the ejected compacts. Preliminary experiments with 20–30 mesh crystalline sucrose indicate that values of die reaction, F_d, and ejection force, F_e, increase with increased applied pressure. For a given compaction pressure, F_e and F_d decreased as the rate of application of the compacting force increased. For the range of rates available F_d was independent of the rate of movement of the die. The shear strength of ejected compacts prepared at a constant pressure increased with increase in the rate at which the shear force was applied.

THE compression of powders in cylindrical dies has been studied by several workers (Duwez & Zwell, 1949; Spencer, Gilmore & Wiley, 1950; Ballhausen, 1951; Sheinhartz, McCullough & Zambrow, 1954; Toor & Eagleton, 1956), and it is claimed that their results, in general, conform to the exponential relationship:

$$\log_e F_a/F_b = 4\mu\eta L/D \quad .. \quad .. \quad .. \quad (1)$$

where F_a and F_b are the applied and transmitted forces respectively, in a compact of length L and diameter D. The coefficient of friction μ, and the ratio of radial to axial stress, η, have been assumed to be constant.

It has been pointed out already (Train, Carrington & Hersey, 1962) that control of relative interfacial movement between material being compacted and the die wall is important. An apparatus was devised which measured the axial compressive forces and provided a means by which all movements of the test material and top and bottom punch faces could be measured relative to the die.

Reproducible values for die wall reaction could only be obtained in the pressing of solid cylinders of polymers when a minimum relative movement between plug and die wall was induced. Using a similar technique Hersey (1960) was able to show that the Bowden & Tabor (1954) theory of friction could be applied to a compacting system.

In the previous work (Hersey, 1960; Train & others, 1962; Train & Hersey, 1962) the increase in axial force and the movement of the die was incremental, with the possibility of variations in rate of loading and rate of die movement. A modified form of apparatus is now described which permits continuous consolidation of the test material at a fixed speed whilst the die is moved at a constant rate, the compressive force, the die reaction, and the change in length of the compact being recorded during

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the process. Provision of suitable controls enables the rate of compressive loading and the rate of die movement to be varied independently of each other.

APPARATUS

A Tangye four column, 100 ton, hydraulic press was used as the primary ram necessary to compact the powder fill. The secondary hydraulic ram, which moved the die relative to the compact, was mounted on the lower platen of the primary ram and the remaining apparatus was assembled between the top platen of the main press and the secondary ram, in the manner of Train & others (1962), as shown in Fig. 1. The

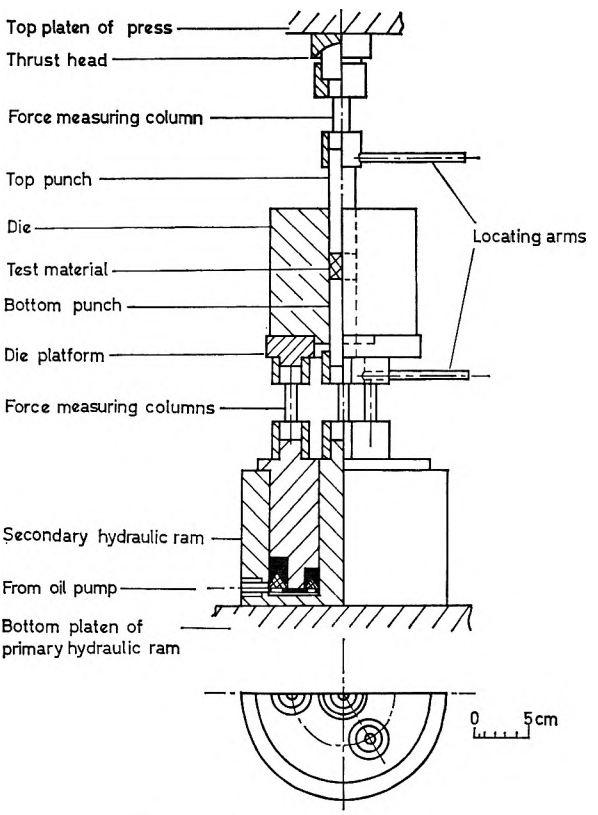

FIG. 1. Die pressing apparatus.

die of hardened A13 steel (Edgar Allen and Co. Ltd.) was 15 cm diameter and 15 cm long, and had a uniform, circular, bore which was chrome plated and ground and polished to a final diameter of 2·41 cm. To minimise flow of test material around the punch faces the clearance at the punch tip was 0·0005 cm. This surface was tapered back for 0·5 cm

to give a clearance of 0·005 cm which was maintained for the remaining length of the punches.

Provision was made for raising the lower platen of the press by means of a hand pump, or continuously by means of a pump (Type IH 036, Chamberlain Industries Ltd.) driven by a 1·5 h.p. motor running at 1,450 rpm. The piston of the secondary ram was raised by a similar pump connected to a 0·75 h.p. motor.

The hydraulic circuit for each ram (Fig. 2) was a separate entity enabling individual control. In each circuit the application and release of pressure was controlled by Dowty single bank valves (Type CV2/O3S/BZ) and the rate of movement of each ram was governed by Vickers

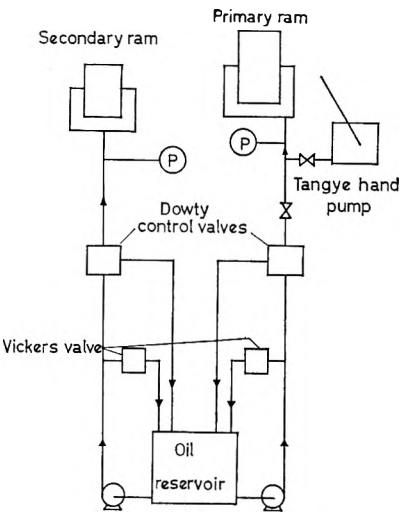

FIG. 2. Hydraulic circuit.

flow control valves (Type FG-02-250-M, Stein Atkinson Vickers Hydraulics Ltd.). By this means it was possible to vary at will the time of application of compressive load on the powder fill, the rate at which the compressive load was applied, and the time and rate of movement of the die relative to the compact.

Force measurements were made by utilising elastically strained pillars on which were bonded resistance strain gauges, enabling the reaction to be measured electrically. The measurements were recorded continuously during a pressing operation using an Ultraviolet Recorder (New Electronic Products, type 1050) with its associated carrier amplifier (New Electronic Products, type 1070).

Relative movements between the punches and die were measured ($\pm$0·01 mm) by using suitably placed dial gauges. The initial depth of powder in the die was determined by measuring the distance between location marks on the top and bottom punches. Since the compressive load was applied continuously at constant rates, the pointers on the dial gauges moved rapidly. Readings of punch positions were taken by using

a "Robot Junior" camera to photograph the gauges at 1/500 sec at *f*11 on 35 mm FP3 Series II film. As each photograph was taken the event was indicated on the instrument recording the forces, so that the height of the compact could be related to the pressure applied at that instant.

The compaction force can be applied at rates varying from 344–860 kg/sec. The rate at which the compaction force was applied was computed from the paper recording the force measurements. Since the paper issued from the recorder at a known and constant rate, the time taken for the compacting force to increase from zero to its maximum value was obtained by measuring the paper. The rate of loading is then the maximum force divided by the time taken to attain the maximum value.

Free movement of the die could be varied between 0·1–0·25 cm/sec. However, owing to the mechanism of action of the Vickers flow control valve, large die wall forces stopped the movement of the die at low rates of movement. Consequently the practical range of movement was limited to 0·15–0·25 cm/sec.

Experimental

GENERAL METHOD OF OPERATION

The bore of the die was cleaned, polished, and degreased with a mixture of equal parts acetone and carbon tetrachloride before use. The punch tips were lubricated with a film of colloidal graphite, deposited from an acetone "dag" suspension (Acheson Colloids Ltd.).

For each experiment the apparatus and test material were assembled as in Fig. 1. During the compression cycle the applied force, F_a, and the die reaction, F_d, were recorded continuously and photographs of the dial gauges were taken at frequent intervals. When the desired pressure level had been attained the compaction was stopped, the pressure released immediately, the lower punch removed from the die, and the compact ejected by moving the die upwards relative to the stationary upper punch. The ejection force, F_e, was recorded. 5 g samples of unlubricated crystalline sucrose 20–30 mesh, stored for not less than 7 days over silica gel in a desiccator, were used throughout this work.

APPLICATION TO THE MEASUREMENT OF SHEAR STRENGTH

In certain instances the shear strength of the ejected compacts was measured immediately after compaction, using a punch penetration method previously applied to homogenous solid specimens (Train & Hersey, 1960). The shear force was applied at a constant rate by using the motor-driven secondary ram, the force necessary to cause shear being recorded during the test; a typical recording of pressure changes occurring during the measurement of shear strength is shown in Fig. 3.

EFFECT OF APPLIED PRESSURE

5 g samples of sucrose were compressed at pressures up to 9,500 kg/cm^2 with the die moving relative to the compact at a constant rate throughout

the operation. During the compaction, measurements were taken continuously of the force acting on the upper punch, F_a, the die reaction, F_d, and the change in position of the punches. The ejected compacts were weighed, and the length and diameter were measured with a micrometer gauge before determination of the shear strength.

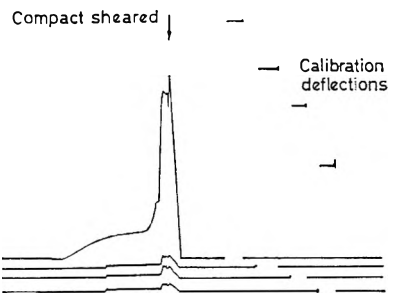

FIG. 3. Pressure changes during the measurement of shear strength.

EFFECT OF RATE OF APPLICATION OF COMPACTING PRESSURE

5 g sucrose was compressed to a predetermined pressure level, applying the compacting force at the slowest rate possible. The compact was ejected from the die and the shear strength determined. Further samples were compressed to the same pressure level but the force was applied at different rates. For each rate of loading F_a, F_d, F_e, and change in density of compact, were measured.

EFFECT OF DIE MOVEMENT ON VALUES OF F_d

5 g samples of sucrose were compressed to a pressure level of approximately 4,300 kg/cm^2. For each sample the die was moved at a different rate and values of F_d were measured during the compaction cycle.

INFLUENCE OF RATE OF APPLICATION OF SHEAR FORCE ON THE OBSERVED SHEAR STRENGTH

Compacts were prepared at a fixed pressure of 1,547 $\pm$ 10 kg/cm^2 from 5 g samples of sucrose, the rate of application of compacting force and all other conditions being maintained the same for each compact. The shear strength of the compacts was then measured applying the shear force at different rates.

Results and discussion

The results are presented in Tables 1–3, and Figs 4–6.

It was observed that the ejected compact bore lamination marks in the horizontal plane when compressed at pressures of 2,852 kg/cm^2 and higher. Sometimes the compact was whole with horizontal cracks visible, at other times the various layers were quite loose and capable of easy separation. There appeared to be no definite correlation with the

applied pressure but the layers were particularly loose at pressures of 2,852–6,350 kg/cm².

The length of the ejected compact was not reduced below that at 2,605 kg/cm² compaction pressure until P_a = 7,450 kg/cm² (Table 1), at which pressure the number of laminations in the compact were fewer and the upper laminations were particularly highly compressed and becoming translucent in appearance, indicating a consolidated mass with fewer internal reflecting surfaces.

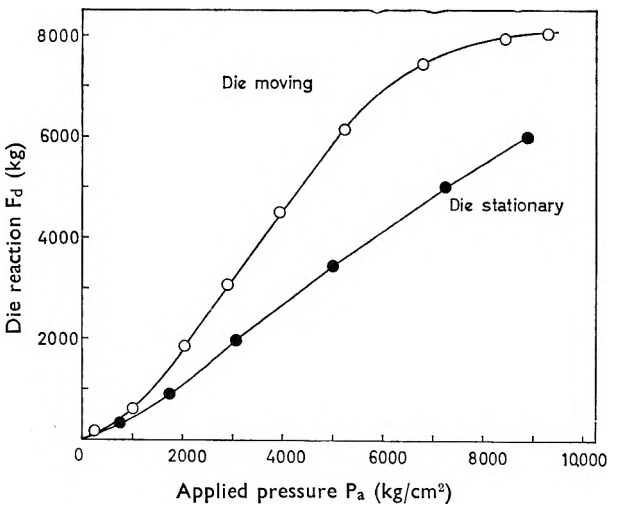

FIG. 4. Effect of applied pressure on die reaction.

TABLE 1. THE EFFECT OF APPLIED LOAD ON THE COMPACTION OF SUCROSE 20–30 MESH

Length of compact cm	Diam. of compact cm	Max. P_a kg/cm²	Max. F_d kg	Ejection force, F_e kg	Relative* density ρ_R	Shear strength kg/cm²
0·821	2·42	884	763	—	0·856	91·5
0·780	2·42	1,198	773	134	0·900	135
0·765	2·42	1,752	901	262	0·919	211
0·745	2·43	2,151	1,873	360	0·943	235
0·742	2·43	2,605	1,851	495	0·948	253
0·754	2·43	2,852	2,670	760	0·933	224
0·752	2·42	3,338	2,914	701	0·934	252
0·753	2·42	4,193	4,160	879	0·934	259
0·746	2·42	5,097	5,199	1,751	0·943	310
0·745	2·42	6,350	6,407	1,128	0·943	295
0·741	2·42	7,450	7,484	2,465	0·949	345
0·749	2·42	8,050	7,983	2,893	0·938	353
0·740	2·42	9,895	8,371	—	0·950	281

* Relative density is calculated from dimensions of extruded compact.

Values of F_d only begin to approach a maximum (Fig. 4) at the highest pressure used. Since the force lost to the die wall is unlikely to reach a maximum until the area of the compact—die wall interface is a maximum, i.e., at zero porosity (ρ_R = 1), the explanation lies in Fig. 5 where it is seen that this degree of consolidation is only attained at pressures greater than 7,250 kg/cm².

The relative density, ρ_R, is defined as the ratio of compact density to density of the solid material. Curve A of Fig. 5 shows the density of the compact under load and Curve B indicates the densities of the ejected compacts. The maximum relative density attained in the ejected compacts was 0·95 and was achieved first at an applied pressure of 2,600 kg/cm²; compaction to greater pressure levels did not decrease the porosity further. It is apparent also that the compacts compressed to pressures

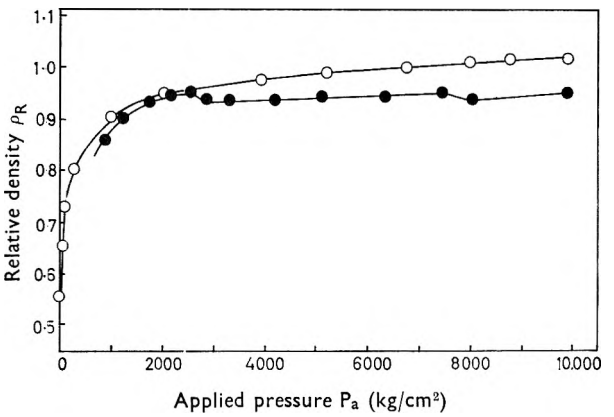

FIG. 5. Effect of applied pressure on density of compact. ○ Compact under load (Curve A). ● Ejected compacts (Curve B).

greater than 2,600 kg/cm² undergo appreciable axial expansion when ejected from the die.

The pressure at which lamination of the compact was apparent is that at which the ejected compact approached its maximum density. Pressing beyond this point is equivalent to the pressing of a solid body, serving only to increase the elastic strains residing in the compact when the applied pressure is removed. It is not known if these laminations are present after removal of the applied load and before ejection of the tablet.

The ejection force, F_e, increased with increased compaction pressure (Table 1). However, at a given pressure F_e and F_d decreased as the rate of application of the compacting pressure was increased (Table 2). At

TABLE 2. THE EFFECT OF RATE OF APPLICATION OF COMPACTING PRESSURE

Rate of applying load, kg/sec	Max P_a kg/cm²	Max F_d kg	F_e kg	Shear strength kg/cm²	a ρ_R	b ρ_R
344	1,779	1,319	586	201	0·95	0·91
534	1,772	972	436	224	0·97	0·91
677	1,755	708	249	230	0·98	0·91
826	1,760	651	233	234	0·99	0·91
860	1,797	698	258	221	1·02	0·91

a. Value when under load of Max P_a.
b. Value calculated from dimensions of ejected compact.

the same time values of ρ_R increased with increased rate of application of pressure, although the density of the ejected compacts was unaffected.

The compaction force was followed by observation of a Bourdon gauge which registered the oil pressure in the primary hydraulic ram. Owing to the rate at which the applied pressure increased it was difficult to stop the compaction process at exactly the same pressure level in each compression, and this accounts for the observed variations in the maximum value of P_a recorded in Table 2. It is considered that the small variations in the magnitude of P_a will have a negligible effect on the experimental results, compared with the effect of changing the rate of application of pressure.

Values of F_d were greater when the die was moved at constant rate than when maintained stationary (Fig. 4). For the rates of movement available, the die reaction was independent of the rate of die movement. Relative movement between the powder and the stationary die as the material consolidates under an applied pressure is not uniform along the length of the compact; the amount of movement near to the lower punch is less in a single-ended pressing than near the face of the upper punch. Movement of the die increases the number of powder-die contacts which are sheared, and this is reflected in an increased value of F_d.

OBSERVATIONS ON THE SHEAR STRENGTH OF SUCROSE COMPACTS

The shear strength of the ejected compacts increased with increased applied compaction pressure (Fig. 6), approaching a plateau as the compact assumed minimum attainable porosity. This plateau represents the maximum shear strength of a coherent compact; thereafter the values represent the strength of the most densely compacted upper laminations.

For a given compaction pressure, increasing the rate at which the compaction force is applied increased the shear strength of the compacts (Table 2). In addition the observed shear strength of compacts prepared at a constant pressure of $1{,}547 \pm 10$ kg/cm^2 increased as the rate of application of the shearing force increased (Table 3). The latter results are in agreement with other determinations of "strength", e.g., tensile strength, compressive strength, crushing resistance of tablets, in that they are influenced by the rate at which the load necessary to cause failure is applied.

TABLE 3. THE EFFECT OF RATE OF SHEAR ON SHEAR STRENGTH

Mean compaction pressure: 1,547 kg/cm^2

Rate of shear kg/sec	Observed shear strength kg/cm^2
23	182
29	189
166	201
308	223
432	205

CONCLUSIONS

On the basis of these preliminary results it is concluded that a standard operating procedure should be adopted when comparing the behaviour

of different materials. For convenience of operation the following conditions were chosen for later work:

1. Rate of application of compacting pressure to be constant at 638 kg/cm^2 per sec.

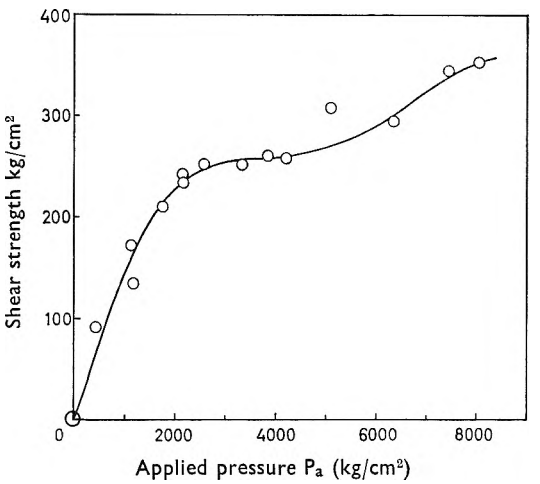

FIG. 6. Effect of applied pressure on shear strength of compact.

2. The rate of die movement to be constant at 0·22 cm/sec.

These operating conditions influence each other. If the die moves too quickly it will reach the top of the upper punch before maximum pressure is attained. The conditions decided on allow die movement throughout the entire compaction stage even when pressing to high pressure levels.

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Automatic apparatus for recording duration of narcosis in mice

A. L. A. BOURA, E. J. R. HARRY AND B. D. LEWIS

An apparatus for automatically recording the duration of narcosis in mice is described. Following induction of anaesthesia the animals may be left unattended for the remainder of the experiment.

THE effects of compounds on the duration of drug-induced narcosis in mice are commonly studied during their preliminary examination for possible actions on the central nervous system. The test, often referred to as the "sleeping time" test, was first described by Winter (1948) and is based on findings that central depressants prolong and central stimulants reduce the duration of anaesthesia caused by a standard dose of hypnotic (usually pentobarbitone or hexobarbitone sodium).

As usually carried out the test is subject to a number of disadvantages. Each experiment requires the undivided attention of the experimenter to record the time that elapses from the moment of induction of anaesthesia to that of recovery for each mouse. In addition, errors arising during determination of the time of recovery, often the return of the righting reflex is used as the end-point, adversely affect precision. These arise because of the premature disturbance of the mice, by the handling necessary for the assessment of the end-point and also by the recovered animals running across their neighbours before capture. Furthermore, as determination of the end-point is a subjective assessment it varies with the skill of the observer. It was of interest therefore to devise an apparatus* that recorded the duration of anaesthesia automatically using an objective end-point the mice being kept in a uniform environment free from extraneous influences. We describe here the construction of such an apparatus and compare the results obtained with its use with those obtained using the established, conventional technique.

Method

The principle of the method is that the duration of anaesthesia in each mouse is recorded separately by a timing device, started by closure of a microswitch under the weight of the mouse placed on a small spring loaded shelf. The timer is switched off by the mouse falling from the shelf during recovery from the central depressant effects of the barbiturate.

The design of the shelf and its position relative to the micro-switch is indicated in Fig. 1. Following intravenous induction of anaesthesia each animal is placed on a hinged perspex shelf S, pivoted at P the animal's weight depressing the shelf against the spring loaded contacts of the microswitch M. The incoordinated movements that occur during the preliminary stages of recovery from the barbiturate are used as the end-point

From the Pharmacology Laboratory, Reckitt & Sons Ltd, Hull.

* Demonstrated to the British Pharmacological Society Meeting, 2–4th January, 1964.

for recovery. These cause the mouse to fall from the shelf, which is only just large enough to support it, allowing the microswitch to reopen, thus breaking the circuit. Small side walls are fitted to each shelf so that the mouse does not fall because of its respiratory activity and has to give a very positive movement in order to fall off.

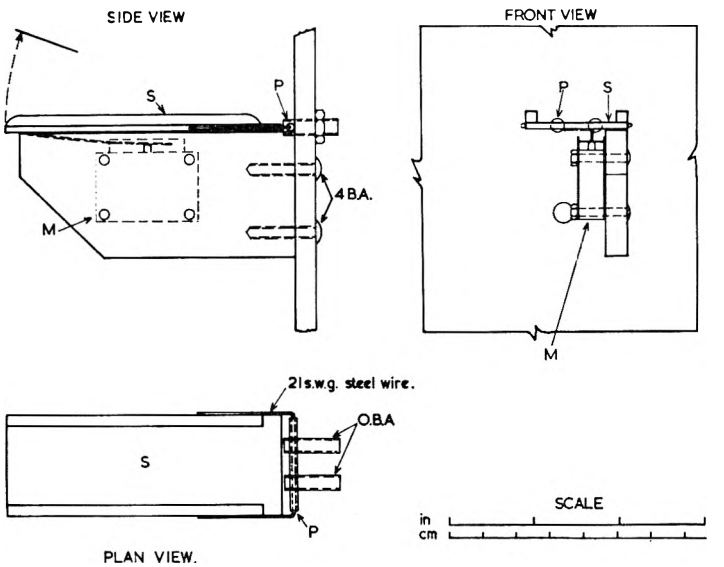

FIG. 1. Working diagram of a shelf and its accompanying microswitch. The shelf S pivoting at the point P is illustrated in its working position depressing the lever of the microswitch M.

The shelves and their accompanying microswitches are mounted on a perspex stand 9 cm apart, in rows of 5. Each row is 15 cm above and 9 cm in front of the row below. This arrangement minimises the disturbance to other animals caused by a mouse falling from its shelf. A gauze net placed beneath the shelves prevents injury. The entire unit is installed in a thermostatically controlled incubator, as the precision of the test is increased by carrying it out in a uniform and constant temperature (Riley & Spinks, 1958).

THE ELECTRONIC CIRCUIT

The simple electronic circuit is shown in Fig. 2. The contacts P of a Palmer time clock are adjusted to close briefly once every 5 sec allowing relay A to energise, closing momentarily contacts A_1. When the mouse is placed on its shelf the microswitch contacts M adopt the position indicated in the diagram. The manually operated switch S is then momentarily closed energising coil "a" of the magnetic latching relay B so closing contacts B_1 and B_2. This allows passage of the electrical impulses caused by closing contacts A_1 to pass through contacts B_1 triggering a Post Office electromagnetic counter C. When the mouse falls from the shelf, the

contacts M change over causing the next impulse to pass through the "suicide" contacts B_2 to the reverse wound coil "b" opening contacts B_1 and B_2. The counter thus indicates the number of 5 sec periods that

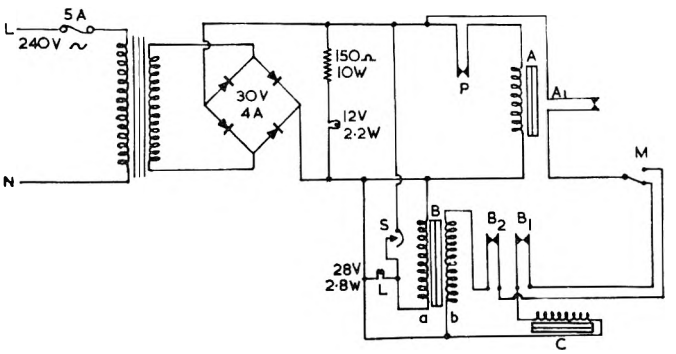

FIG. 2. Simplified circuit diagram. The circuit utilising the components L, S, B, C and M is repeated in parallel for each shelf in the apparatus. The current supplied by the full wave selenium rectifier is sufficient for up to 50 shelves and their associated electromagnetic counters. P are the contacts of a Palmer Timer type B.116. A is a P.O. 3,000 type relay having a 2,000 Ω coil; contacts A_1 rated at 10 A close on energising. B is a magnetic latching (remanence) relay having two coils a and b of 400 Ω and 1,000 Ω respectively causing opposing magnetic fluxes. C is a P.O., four digit, electro-magnetic counter (2,000 Ω coil). M is a Bulgin microswitch type 'M' list No. S520/W. S is a "push to make" switch, L a pilot light.

elapse during anaesthesia. Until S is again closed to reset the circuit, any subsequent closure of the microswitch does not activate the counter preventing resumption of recording should a recovered mouse climb back on to a vacated shelf.

Results

The relative accuracy of data obtained using the apparatus has been assessed by comparing the results from its use with those obtained from similar experiments carried out concomitantly under conventional conditions. The latter were carried out as follows. Groups of mice received either one of a series of doses of the drug under test, or saline as a control, 30 min before administering intravenously an anaesthetising dose of barbiturate. Immediately following induction of anaesthesia the animals were placed in rows on a thermostatically controlled heated tray and the return of the righting reflex taken as the end-point for recovery.

Statistical analysis was separately carried out on the data obtained using each method. On all comparable sets of data the within drugs (residual) term was used as a measure of experimental variability. Fig. 3 shows that using either the automatic apparatus or the heated tray the narcosis times are log normally distributed, as the cumulative distributions conform closely to straight lines when plotted on logarithmic probability paper, confirming the results of Winter (1948). The ratio of the geometric means of the narcosis times indicated by the automatic apparatus,

for the drug-treated and control groups respectively, provides therefore an adequate initial measure of the degree of central effect.

To compare the variability of the results obtained from the automatic apparatus with those obtained using the tray, analyses were carried out

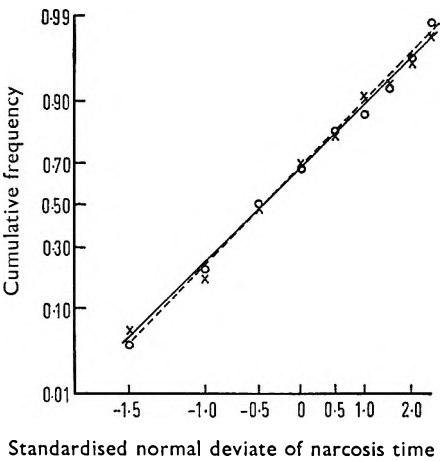

FIG. 3. Cumulative frequency distributions of narcosis times obtained using either the heated tray or the automatic apparatus. ×—× Heated tray. ○ - - - ○ Automatic apparatus.

on automatic apparatus and tray results separately and the residual mean squares used as an estimate of the within experiment variances. These were then tested for significance (Table 1) and in only one instance (60 mg/kg leptazol followed by 70 mg/kg pentobarbitone sodium) was a significant difference obtained ($P = 0{\cdot}05$), the automatic apparatus variance being less than the tray variance. Individual variances, calculated from all data obtained using either the automatic apparatus or the heated tray, were separately tested for homogenity, in order to obtain an overall estimate of the experimental variance using either technique. The variance obtained from the experiment using 2·5 mg/kg strychnine followed by 65 mg/kg pentobarbitone sodium was found to be significantly different from the others and therefore omitted from the overall variances. The ratio of the two overall variances (1·06) indicates that there is no significant difference between variation using the automatic apparatus and variation using the tray.

Table 2 shows that comparable levels of significance are obtained from an analysis of variance of figures obtained using either method and gives comparisons of the mean square ratios for the source of variation between doses. The results appear to be reasonably consistent indicating that the apparatus can distinguish between doses as well as the heated tray. It also indicates that although small groups of mice (7–10) are sufficient for screening purposes, the size of the group must be increased for accurate comparisons of centrally acting drugs.

TABLE 1. NARCOSIS TIMES OF MICE AFTER ADMINISTRATION OF CENTRALLY ACTIVE DRUGS COMPARING DATA OBTAINED USING THE HEATED TRAY METHOD WITH THAT USING THE AUTOMATIC APPARATUS. (GROUPS OF 7–10 ANIMALS)

Drug	mg/kg s.c.	mg/kg i.v. pentobarbitone sodium	Log mean narcosis time (sec)				Residual mean square		
			Automatic apparatus		Heated tray		Automatic apparatus	Heated tray	Ratio
			Control group	Drug group	Control group	Drug group			
Leptazol	60	70	3·7376	3·6781	3·6187	3·6460	0·00645	0·01608	2·49
,, ...	120	70	3·6963	3·6887	3·7493	3·6015	0·01669	0·01432	0·86
,, ...	240	70	3·8520	3·6838	3·8313	3·6048	0·00920	0·01449	1·57
Bemegride	10	75	3·5479	3·5962	3·6508	3·6126	0·02038	0·01765	0·87
,, ...	10	75	3·6336	3·6231	3·7335	3·6087	0·01130	0·00768	0·68
,, ...	15	75	3·5898	3·5299	3·7376	3·6780	0·00836	0·00913	1·09
Strychnine nitrate	2·5	65	3·6525	3·2970	3·6830	3·4679	0·06435*	0·06127*	0·95*
,, ,, ...	2·5	70	3·6940	3·6377	3·6936	3·6356	0·01580	0·01354	0·86
,, ,, ...	2·5	75	3·7669	3·7344	3·8352	3·8573	0·03105	0·02444	0·79
Chlorpromazine HCl ..	5·0	60	3·3530	3·7466	3·3680	3·8753	0·00582	0·00798	1·37
Dibenamine HCl	10·0	65	3·6970	3·8047	3·8741	3·9214	0·01387	0·02269	1·64
						Overall variance excluding*	0·013903	0·014801	1·06

Analysis of the log transformed data was also made to determine whether any differences in sleeping time occurred when the mice were placed on various shelves or in various locations on the tray. This was done comparing all positions individually, and also as a rows and columns

TABLE 2. MEAN SQUARE RATIOS FOR BETWEEN DOSES WITH SIGNIFICANCE LEVELS

Drug	mg/kg s.c.	Pento-barbitone sodium mg/kg i.v.	Automatic apparatus		Heated tray	
			Residual degrees of freedom	Mean square ratio	Residual degrees of freedom	Mean square ratio
Leptazol .. .	60	70	16	2·47 N.S.	17	0·22 N.S.
,, .	120	70	14	0·01 N.S.	18	7·62 S.
,, .	240	70	14	12·10 H.S.	17	16·77 V.H.S.
Bemegride .. .	10	75	16	0·51 N.S.	18	0·42 N.S.
,, .	10	75	16	0·04 N.S.	18	10·13 H.S.
,, .	15	75	16	1·93 N.S.	18	1·95 N.S.
Strychnine nitrate .	2·5	65	16	8·84 H.S.	18	3·78 N.S.
,, .	2·5	70	15	0·85 N.S.	17	1·18 N.S.
,, .	2·5	75	16	0·15 N.S.	18	0·10 N.S.
Chlorpromazine HCl	5·0	60	16	198·65 V.H.S.	18	161·27 V.H.S.
Dibenamine HCl ..	10·0	65	16	3·77 N.S.	18	0·49 N.S.

N.S. = Not significant $P > 0.05$
S. = Significant $P < 0.05$
H.S. = Highly significant $P < 0.01$
V.H.S. = Very highly significant $P < 0.001$

analysis. No significant differences were found, indicating that the results obtained are not affected by the location of the mouse within the cabinet or on the tray.

Acknowledgements. Our thanks are due to Mr. D. L. Stephenson and Miss M. Holden for help in preparing the illustrations.

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A simple liquid flow recorder

LEE AI-SENG AND ROBERT C. Y. LIN

A liquid flow recorder of simple design which can be easily constructed with readily obtainable material at low cost is described. It is used with a piston recorder and a time interval marking clock to measure the rate of liquid flow over a range of a few drops to 50 ml/min.

THERE are several types of liquid flow recorders used in physiological laboratories: that described by Haranath (1962); Stephenson's flow recorder (Burn, 1952); Gaddum's drop recorder (1953); Thorp's impulse counter (1953); Rothlin's drop counter (1960); Fleisch's (1960) totalisator; a simplified recording bubble flow meter for measuring blood flow by Hammond, Hyman & Nelson (1950) and a volume recorder devised by Bülbring, Crema & Saxby (1958).

The shortcomings of these recorders are: (i) the simple types of Haranath, Stephenson and Gaddum lack range and therefore can be used to measure only a slow rate of flow; (ii) excepting the recorders of Hammond and Bülbring, all the others do not furnish quantitative data of the rate of flow and (iii) the advanced types are elaborate in design and expensive to construct. We present here a simple recorder which can be constructed with ordinary material and parts at low cost.

DESIGN AND SPECIFICATION

The present liquid flow recorder consists of three units: (1) a piston recorder (Palmer B126); (2) an A.C. time interval marking clock (Palmer C50) and (3) a glass vessel with accessories mounted on a wooden block. The whole assembly of the recorder in use is shown in Fig. 1. Items 1 and 2 are standard equipment usually available. The third item can be constructed from the parts as shown in Fig. 2. "A" is a glass vessel constructed with a side tube near its upper end for connection to a piston recorder; "B" is a glass funnel about 3 in in diameter for the collection of liquid inflow. This is fixed to the upper opening of glass vessel "A" (see Fig. 1). The stem of the funnel is about 5 cm long and its lower opening is constricted to about 1 mm in internal diameter to convert the lower opening into an inlet liquid valve. This constriction allows the liquid flowing through it by means of capillary action, to effect a "liquid seal". This enables the piston recorder to respond to the changes in pressure within the glass vessel "A" after liquid has entered it. "C" is soft rubber tubing, about 5 cm long and 5 mm in diameter, attached to the lower end of glass vessel "A". The type used in reservoirs in fountain pens was most suitable. "D" is a lever made of perspex, one end of which is bent into a hook. This has a hole of 5 mm in diameter drilled through it at the position shown, to allow passage of the soft rubber tubing (see Fig. 2). At the other end of the lever is mounted a nut "N"

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of about 20 g weight. The function of the lever is to close the soft rubber tubing with the hook end by the weight of the nut, thus preventing the liquid in the glass vessel "A" from flowing out. "S" is a solenoid of 500 ohms resistance, which, when magnetised by a momentary flow of

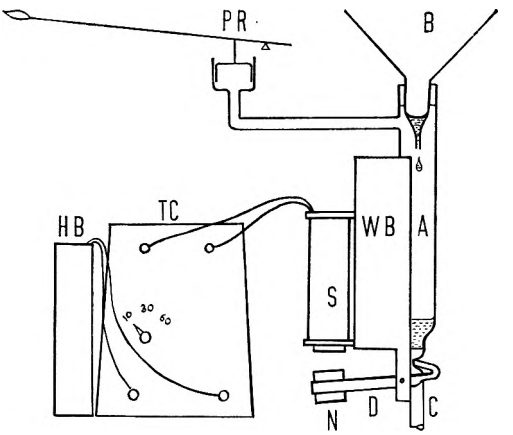

FIG. 1. Assembly of the liquid flow recorder composing of the following parts: A, glass vessel; B, a glass funnel; C, rubber tubing; D, perspex lever; HB, high tension battery; PR, piston recorder; S, solenoid; TC, time clock; WB, wooden block, and N, bolt nut.

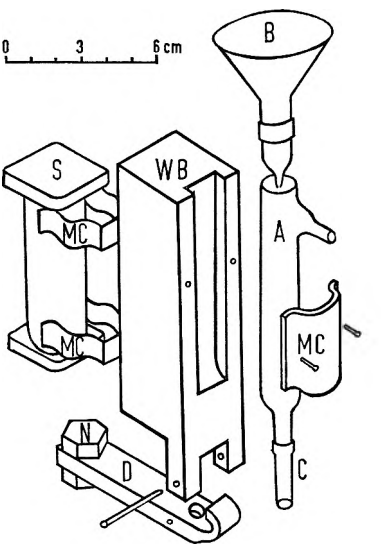

FIG. 2. Breakdown parts of the liquid flow recorder; A, glass vessel; B, a glass funnel; C, rubber tubing; D, perspex lever; N, bolt nut; MC, mounting clip; S, solenoid and WB, wooden block.

current produced by the time clock closing the circuit, raises the nut "N" allowing the Perspex hook to move downward thus relieving the pressure

on the rubber tubing. The liquid collected in the glass vessel "A" then flows out. "WB" is a wooden block for the mounting of "A", "S" and "D" together. "MC" are three metal clips. A breakdown of the design of the recorder into its various parts is shown in Fig. 2.

OPERATION

Before the recorder is used, a piston recorder of suitable size is chosen and connected to the side tube of glass vessel "A" by rubber tubing. A 45 V dry battery is connected to the solenoid through the signal posts of a time clock. The time interval for the time clock to operate the solenoid circuit is selected on the basis of the slower the rate of liquid flow to be measured, the longer the time interval.

The liquid is collected by funnel "B". On entering glass vessel "A", it raises the air pressure therein and causes the writing lever of the piston recorder to rise. The rise in height of the lever is proportional to the volume of liquid entering the vessel "A". The height recorded by the lever per unit time is thus a direct measure of the rate of liquid flow. A calibration scale of the height in terms of the rate of flow in volume/min should be made before the operation of the recorder. An example is shown on the left-hand side of Fig. 3 which is a tracing showing changes in the rate of outflow of perfusate coming out from the blood vessel of

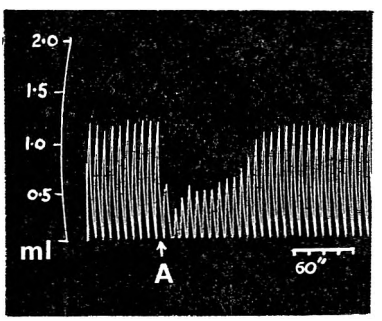

FIG. 3. Kymograph tracing showing changes in the rate of liquid-flow through the blood vessels of a perfused isolated rabbit ear as affected by the injection of 0·25 μg of adrenaline at A to the perfusate made with the present recorder. A calibrated scale of the rate of flow per 30 sec is shown on the left-hand side. Time is in 60 sec.

an isolated rabbit ear as affected by the injection of 0·25 μg of adrenaline. The downward stroke of each tracing registers the draining out of the collected liquid from glass vessel "A" at an interval set by the time clock and performed by the action of the solenoid "S".

DISCUSSION

We find the present flow recorder offers the following advantages: (1) the main unit can be easily constructed from readily obtainable materials at low cost; (2) the tracing obtained shows at a glance, changes in a

positive way the rate of liquid flow and also gives a quantitative measure when referred to the calibration scale; (3) it offers a fairly high sensitivity of response which is reproducible and (4) by varying the glass vessel "A", and the piston recorder size and the time clock setting, the range of the recorder can be extended from a few drops to 50 ml/min.

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Letters to the Editor

A restraining cage for metabolic studies in rats

SIR,—The cage shown in Fig. 1 was developed to enable urine and bile to be collected from rats after oral administration or injection of drugs or other foreign organic compounds. While similar to the cage described by Bollman (1948), it has the advantages of being easily and rapidly adjustable to rats of different sizes and weights and of allowing urine to be collected normally.

The cage is made of 8 mm thick Perspex and consists of an 18 by 35 cm base plate and three 10 cm wide upright pieces. The front piece is 27 cm high and has a spring clip to hold a water bottle. The middle piece is 12·3 cm high and is placed 6 cm from the front piece. Both of these are screwed to the base plate and are grooved to take a slidable food cup made of 3 mm thick Perspex. The end piece including its base is 22 cm high and is movable along a slot in the base plate. The end piece can be moved from 13 to 25 cm from the front piece and is fixed by a set screw. Holes for the drinking tube and the tail of the rat are 2 cm in diameter.

The urine collector, made of stainless steel sheet, is 8 cm long and is placed 3 cm under the floor bars on the end plate. It slopes downward and narrows towards the outlet tube which is 8 cm under the floor.

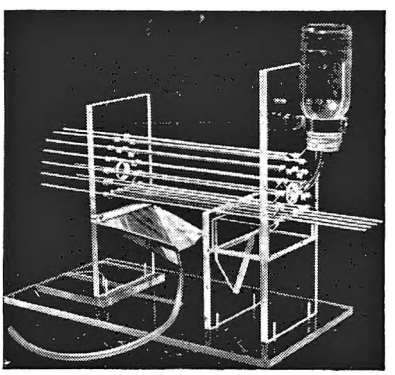

FIG. 1

The floor and sides of the cage are made of 32 cm long stainless steel rods (3 mm diameter welding rods) placed through holes in the upright pieces. The floor is 6 cm wide and consists of six rods placed 12 cm above the base plate. Access to the food cup is obtained by sliding the four inner rods back to the middle upright piece. The sides are formed by rods placed in the outer ring (10 holes, 6 cm high by 6 cm wide) or the inner ring (8 holes, 4·5 cm high by 4·5 cm wide) and the enclosed space can be easily varied by using different combinations of holes. The rods are bent on one end and are prevented from sliding by stretching rubber bands on the opposite ends.

Acknowledgement. I wish to express my thanks to Mr. J. S. Johansson for technical assistance.

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Amphetamine toxity in aggressive mice

SIR,—The toxic effects of amphetamine in mice can be influenced by a number of factors: weight of the animal (Chance, 1947; Fink & Larson, 1962), environmental temperature (Hohn & Lasagna, 1960; Askew, 1961; Fink & Larson, 1962) noise (Chance, 1946, 1947; Cohen & Lal, 1964), the number of animals in a cage (Chance, 1946; Burn & Hobbs, 1957) and painful stimuli (Weiss, Laties & Blanton, 1961).

Mice kept isolated for a long time and showing aggressiveness also show increased sensitivity to the toxic effects of amphetamine.

Male, Swiss, albino mice, weighing about 20 g were used. They were kept usually 6/cage in Makrolon cages with a floor surface of 40 cm^2 at a room temperature of 22° and a relative humidity of 60%.

Aggressive mice were obtained (Yen, Stanger & Millman, 1959) by isolating the animals in individual cages of the same dimensions, but with an opaque wall, for four weeks. After this period, the mice became aggressive and fought amongst themselves when they were grouped.

Dexamphetamine was given intraperitoneally in different doses to both normal and aggressive mice. Each group contained isolated and grouped animals. The toxicity was calculated after 24 hr (Litchfield & Wilcoxon, 1949).

The results are in Table 1.

TABLE 1. TOXICITY OF AMPHETAMINE IN NORMAL AND AGGRESSIVE MICE

Experimental condition	LD50 (and 95% confidence fiducial limits) of dexamphetamine in mg/kg/i.p.	
Normal mice		
— isolated	47·5	(32·7 — 68·8)
— grouped	9·0	(8·0 — 12·0)
Aggressive mice		
— isolated	11·0	(7·3 — 16·5)
— grouped	3·7	(2·6 — 5·3)

A minimum of 48 mice was used for each experimental group.

The toxicity of dexamphetamine is increased in aggressive mice compared with normal mice whether they are isolated or grouped at the moment of the administration of the drug.

Since Halpern, Drudi-Baracco & Bessirard (1962) suggested a correlation between amphetamine toxicity and the level of brain catecholamines, this was investigated, but when brain 5-hydroxytryptamine and noradrenaline was

determined spectrofluorometrically (Shore, 1959) these were the same for normal and aggressive mice.

Our results add another factor to the many already known to affect amphetamine toxicity. They also provide a new lead to the understanding of biological changes occurring during the development of the aggressive behaviour.

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A method for evaluating imipramine-like agents in rats*

SIR,—Demonstration of imipramine-like activity in animals is difficult. Stein & Seifter (1961) reported that the increase in self-stimulation rate induced by methamphetamine was enhanced by imipramine as a result of augmentation of the central adrenergic reward system. We have now investigated the behavioural effects of amphetamine and imipramine in animals trained for an automatic pole-climbing apparatus (Aceto, Kinnard & Buckley, 1963).

A group of six male albino rats (250–350 g) was trained to climb a pole in response to an auditory signal, and so avoid a shock delivered by an electrified grid floor. A successful climb depressed a microswitch which terminated the trial and permitted a longer intertrial period. The apparatus was programmed so that 100 trials could be presented during a 2 hr session. Each trial consisted of 8 sec of auditory tone (avoidance phase), 6 sec of auditory tone plus shock (escape phase) and 58 sec of intertrial time. A shock scanning device was used which energized the grid by scanning at the rate of 3 times/sec. Current was measured by ammeter, and rats were shocked with 500 V at about 3 mA.

Animals were trained to avoid shock in 95% or better of the trials. In the experiments reported here, none of the drug-treatments significantly impaired the ability of the rats to successfully avoid or escape. The avoidance response latency for each rat was recorded by means of a pen polygraph; 16 control days revealed that the rats climbed the pole in an average time of 5·4 sec, with a range of 4·9 to 6·0 sec. Salts of the drugs, imipramine (hydrochloride) and amphetamine (sulphate), were injected intraperitoneally in saline once a week

* Supported in part by a grant from N.I.H.

just before the experiment, with control experiments using only saline injections at a similar time of day twice a week. All drug-treatments were replicated and the data represent the average of two experiments.

TABLE 1. ACTION OF IMIPRAMINE AND AMPHETAMINE ON AVOIDANCE LATENCIES IN AUTOMATIC POLE-CLIMB RESPONSE

	Average group avoidance (sec) for numbers of trials after injection									
	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100
Controls (16 days)	5·3	5·2	5·4	5·7	5·3	5·5	5·4	5·4	5·3	5·3
Imipramine (10 mg/kg) ..	4·8	4·7	5·5	5·3	5·6	5·9	6·0	5·3	5·8	5·9
Amphetamine (0·25 mg/kg)	4·8	3·9*	4·4	5·0	5·4	5·5	5·7	5·4	5·0	5·3
Amphetamine (1 mg/kg) ..	3·3*	2·2*	2·2*	2·4*	3·0*	3·1*	3·3*	3·6*	3·8*	4·0*
Imipramine (10 mg/kg) + amphetamine (0·25 mg/kg)	3·5*	1·6*	1·8*	2·5*	2·8*	3·5*	3·5*	3·6*	3·5*	3·8*

* $P < 0.05$ from controls.

Results, as summarized in the Table, indicate that 1 mg/kg of amphetamine significantly facilitated avoidance response latency and 10 mg/kg of imipramine, a dose which was without effect in this test, potentiated the effects of 0·25 mg/kg of amphetamine into a response obtained when 1 mg/kg of amphetamine was given alone. The method seems suitable for screening imipramine-like agents.

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Non-depolarising neuromuscular blockade by 3α,17α-bis(quaternary ammonium) 5α-androstanes

SIR,—Although many molecular features are known to influence neuromuscular blocking activity and plausible alternative explanations exist (Loewe & Harvey, 1952; Cavallito & Gray, 1960; Waser, 1959, 1962), the potent neuromuscular blocking activity characteristic of certain bisquaternary salts is often envisaged in terms of a "two-point" attachment in which the cationic heads interact simultaneously with separate anionic sites of the acetylcholine receptors on the post-synaptic membrane as first proposed by Barlow & Ing (1948a, b) and Paton & Zaimis (1948, 1949).

Deductions concerning the spatial separation of these anionic sites have been made (Barlow, 1960 and refs cited) in terms of the interonium distance of the decamethonium molecule, since this compound in several species exhibits the highest potency of the homologous polymethylenebis(trimethylammonium) salts, in the belief that maximal potency is a direct reflection of ability to span exactly the gap between two of the sites which are considered to lie in a regular lattice. It was assumed that the decamethonium molecule underwent interaction

in the fully staggered conformation (interonium distance *ca.* 14Å). But doubts about the validity of this assumption arise from conductimetric studies with polymethylene bisquaternary salts (Brody & Fuoss, 1956; Rice, 1956, 1958; Elworthy, 1963, 1964), which indicate that the extended conformation is not favoured in aqueous or ethanolic solution, and from the high potencies present in a number of bisquaternary salts not capable of an interonium distance as large as 14Å (Bovet & others, 1946, 1947; Lüttringhaus & others, 1957; Haining & Johnston, 1962), including the fully rigid toxiferine I (Craig, 1955) with an interonium distance of *ca.* 9·7Å.

In an attempt to reduce the ambiguities inherent in studies employing freely flexible molecules, we examined (Alauddin, 1962) the steroid nucleus as a supporting moiety upon which to append two quaternary ammonium groups in different spatial arrangements. The compounds so prepared were then to be used for an investigation of the importance of interonium distance and cationic head size in neuromuscular blocking activity. Our assumption that such compounds would possess a suitable hydrophilic to lipophilic balance—known to be of importance in neuromuscular blocking activity (Cavallito & Gray, 1960)—was subsequently borne out by the observation that the steroidal alkaloid malouetine possessed activity similar to that shown by (+)-tubocurarine (Janot & others, 1960).

Initially we aimed to synthesise and study pharmacologically a series of 3α, 17α-bis(quaternary ammonium) 5α-androstanes since inspection of models showed the interonium distance in these compounds to fall within the apparently favourable range of 9·2–10·6Å (according to the conformation adopted by ring A), and since the α-configuration of the quaternary ammonium functions was expected to eliminate possible steric hindrance to interaction with the receptors by the β-methyl groups on C-10 and C-13. Recent publications from another laboratory (Biggs, Davis & Wien, 1964; May & Baker, 1963), describing compounds closely related to some prepared by us, has prompted us to report on our work. The relevant compounds studied by us were 3α, 17α-bisdimethylamino-5α-androstane di(methiodide) (I), 3α, 17α-bisethylmethylamino-5α-androstane di(methiodide) (II), 3α,17α-bisdiethylamino-5α-androstane di(methiodide) (III) and 3α,17α-bisdiethylamino-5α-androstane di(ethiodide) (IV), all of which were prepared from 5α-androstane-3,17-dione by reduction to the corresponding 3β,17β-diol, S_N2 displacement of the derived dimethanesulphonate ester by azide ion (*cf.* Henbest & Jackson, 1962), reduction of the resulting 3α,17α-diazido-5α-androstane to the primary diamine and quaternisation with the appropriate alkyl groups.

Table 1 shows the relative potencies of these compounds as tested on different preparations. On the cat and hen gastrocnemius muscle-sciatic nerve preparations, all four compounds showed a duration of action of approximately one

TABLE 1. NEUROMUSCULAR BLOCKING POTENCY

Preparation	Molar potency (+)-Tubocurarine = 100			
	Compound I	Compound II	Compound III	Compound IV
Cat gastrocnemius muscle/sciatic nerve	26·7	83·7	87·4	45·5
Hen gastrocnemius muscle/sciatic nerve	53·4	111·8	116·5	90·9
Frog rectus abdominis muscle ..	45·7	183·5	497·6	211·9
Rat phrenic nerve/diaphragm ..	5·53	8·04	10·7	11·2

half that of (+)-tubocurarine. All exhibited typical non-depolarising activity as shown by the absence of muscular contracture in the hen, potentiation of the block by ether and (+)-tubocurarine in the cat, failure to support a tetanus in the cat, complete rapid reversal by edrophonium in both the hen and cat and by the results obtained with the frog and rat preparations. This is of interest, as decamethonium shows an initial depolarising block in some species, including the cat, and on replacement of *N*-methyl groups by higher alkyl radicals this is converted into a purely non-depolarising block. Only compound III reduced the height of contraction of the nictitating membrane to pre-ganglionic stimulation of the superior cervical sympathetic nerve in the cat, but this was at a dose of 2 mg/kg which is twenty times that required to produce a 50% neuromuscular block in this animal. Although there was no reduction in blood pressure in the anaesthetised cat, all four compounds had a significant inhibitory action on the Trendelenburg preparation and were equipotent with hexamethonium.

Employing enzyme prepared from rat brain (Fenwick & others, 1957) in a modification of the method of Ammon (1933), all four compounds exhibited weak anticholinesterase activity (pI50 values of 4·11 to 4·73; (+)-tubocurarine, 3·05; eserine, 6·68) suggesting that their shorter duration of action in the cat and hen compared to (+)-tubocurarine could be due to a partial inhibition of acetylcholinesterase.

Table 2 shows the ED50, LD50 and therapeutic index of each compound as determined by intraperitoneal injection into mice using the inclined screen method of Thomson (1946) and calculated by the method of Miller & Tainter (1944). Death in all animals resulted from respiratory paralysis.

TABLE 2. ED50 (±S.E.), LD50 (±S.E.) AND THERAPEUTIC INDEX IN MICE

	Compound I	Compound II	Compound III	Compound IV	(+)-Tubocurarine
ED50 mg/kg ..	4·5 ± 0·36	3·58 ± 0·14	2·72 ± 0·32	5·0 ± 0·5	0·3 ± 0·018
LD50 mg/kg ..	6·1 ±0·38	4·25 ± 0·11	3·82 ± 0·3	7·9 ± 1·52	0·51 ± 0·026
Therapeutic Index LD50/ED50 ..	1·36	1·19	1·41	1·58	1·71

An interesting feature of our compounds is, that unlike the polymethylene bisammonium salts (Elworthy, 1964), the interonium distance can be expected to remain virtually constant as *N*-ethyl groups replace *N*-methyl groups. Thus, the observed increase in potency from the *N*-trimethyl compound to the *N*-diethyl methyl compound and subsequent falling off in the *N*-triethyl compound can probably be directly attributed to the variation of the *N*-alkyl substituents. The high potency present in the stereoisomeric 3β,17β-, 3α,17β- and 3β,17α- compounds related to ours (Biggs, Davis, Maxwell & Wien, personal communication, data to be published; May & Baker, 1963), each series having interonium distances which can be expected to differ from the 3α,17α-series, indicates the relative unimportance of a "fixed" interonium distance in determining neuromuscular blocking activity. This not only emphasises the importance that must be attached to other factors such as hydrophilic to lipophilic balance, transport mechanisms and sites of loss, but is of interest in the light of the work of Gill (1959) on ganglion-blocking activity who concluded that potency should fall over a range of interonium distance due to variability in the receptors

(*cf.* also Koshland, 1958). Moreover, the activity of the 3β,17β-, 3α,17β- and 3β,17α-bisquaternary salts makes it clear that there is no steric impedance to receptor interaction by the angular methyl groups on C-10 and C-13 in these compounds and that, as with the malouetine series (Khuong Huu-Lainé & Pinto-Scognamiglio, 1964), stereoisomerism in steroidal bisquaternary salts appears to have little effect on neuromuscular blocking activity. The presence of activity in 3α,17β- and 3β,17α-bis(quaternary ammonium) 5α-androstanes where the quaternary heads lie on opposite sides of the steroid nucleus might perhaps support the adumbration theory (Loewe & Harvey, 1952 *cf.* also Fakstorp & others, 1957) or the receptor pore theory (Waser, 1959, 1962) rather than the "two-point" attachment theory, but the possibility of an edge-on approach of the steroid molecule to the receptor along the staggered chain of nine carbon atoms at positions 3, 2, 1, 10, 9, 11, 12, 13 and 17 cannot be ruled out.

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Determination and identification of amphetamine in urine

SIR,—We report herein an improved and more convenient method than that reported previously, for the determination and identification of amphetamine in urine (cf. Beckett & Rowland, 1964). The determination is based on a modification of the method of Cartoni & Stefano (1963). Gas-liquid chromatography was used with *NN*-dimethylaniline as the internal standard, while amphetamine was identified and separated from related amines as the acetone derivative (cf. Brochmann-Hanssen & Svendsen, 1962).

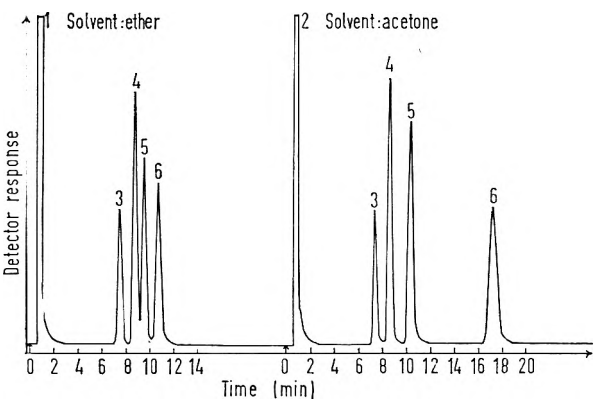

FIG. 1. Chromatograms of amphetamine, methylamphetamine, β-phenylethylamine and *NN*-dimethylaniline in the absence and presence of acetone: 1 ether, 2 acetone, 3 *NN*-dimethylaniline (t_R 7·5 min), 4 methylamphetamine (t_R 8·7 min), 5 amphetamine (t_R 9·5 min in ether, 10·4 min in acetone), 6 β-phenylethylamine (t_R 10·8 min in ether, 17·4 min in acetone).

Procedure. Urine (2-5 ml) was pipetted into a glass-stoppered centrifuge tube, neutralised with dilute hydrochloric acid or sodium hydroxide solution, as appropriate, and then 0·1 ml 5N hydrochloric acid added. The urine was extracted with freshly distilled Analar diethyl ether (3 × 2·5 ml), centrifuged and the ether extract rejected. Sodium hydroxide (0·5 ml, 5N) was added to the urine which was then extracted with ether (3 × 2·5 ml), centrifuging between each extraction. These ethereal extracts were transferred to a 15 ml Quickfit test tube, the base of which was finely tapered. 1 ml of *NN*-dimethylaniline solution in ether (5 μg base/ml) was added and the solution then concentrated on a water bath at 40° to about 50 μl. Approximately 3–5 μl of the concentrate was injected for analysis into the chromatograph. A calibration curve was obtained by measuring the ratio of peak heights of amphetamine to *NN*-dimethylaniline for known concentrations of amphetamine in urine. The curve was found to be linear over the range 0·1–10 μg amphetamine per ml of urine and the method had

an overall accuracy of 100±5%. Interfering peaks were not found on analysing many samples of urine from many subjects.

A Perkin Elmer F11 gas chromatograph was used. The column was a 100–120 mesh acid-washed celite 545, onto which was coated 5% potassium hydroxide, and 10% carbowax 6000, and packed into a 2 metre 1/8 in o.d. stainless steel tube. The column was silizanised with hexamethyldisilizane at the operating conditions which were as follows: column temperature 140°; injection block temperature 250°; nitrogen flow rate 20 ml/min (measured at room temperature); hydrogen pressure 15 lb/in^2; air pressure 25 lb/in.^{2}

Amphetamine was further identified by alteration of its retention time by conversion to its acetone derivative as follows. Acetone (0·5 ml) was added to the ether concentrate in a tapered test-tube and evaporated to about 50 µl on a water-bath at 60°. The concentrate (3–5 µl) was introduced into the chromatograph; a characteristic shift of the amphetamine peak to the amphetamine: acetone derivative peak was observed (see Fig. 1). The observed shift differentiates amphetamine from other related amines chromatographed in the presence and absence of acetone, e.g. see Fig. 1.

The total time necessary for the analysis of sixteen samples is about 6 hr, which is shorter than that for the previous method (Beckett & Rowland, 1964). Furthermore less sample is required and assays of 0·1 µg amphetamine base per ml urine may be made without interference from constituents in urine.

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November 17, 1964

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The pharmacology of hippocampal neurones

SIR,—Little is known of the direct response of cells in the hippocampal cortex to potential cerebral neurotransmitters. We have applied various drugs microelectrophoretically into the environment of these cells and tested their effects on cell firing. This letter reports some preliminary findings.

Our experiments were made on nine adult cats under chloralose-urethane or pentobarbitone anaesthesia. The overlying neocortex was sucked away and the lateral ventricle was filled with 3% agar, and the exposure of the dorsal hippocampus completed. The activity of cells in the CA1 and CA3 fields was recorded extracellularly with the central 2·7 molar NaCl filled barrel of a 5-barrelled glass micropipette. The total tip diameter was 4–8µ. Cell firing was monitored continuously on an oscilloscope and with an audioamplifier. The spikes were also led through a discriminator to a ratemeter the output of which was led to a penwriter.

The other 4 barrels of the microelectrode contained strong aqueous solutions of ionizable drugs. These were expelled from the electrode tip by the passage of appropriate currets.

Over 150 cells have been detected—these either fired spontaneously or could be excited by L-glutamate (confirming unpublished observations by Andersen,

Crawford & Curtis, cited in Crawford & Curtis, 1964). The application of L-glutamate often resulted in the development of high frequency synchronised activity (30–50/sec) making unit recording difficult. γ-Aminobutyric acid had a marked depressant action on all the cells tested. As in the necortex these substances had a characteristic short latency before their effects were observed and their effects ceased within a second of turning off the expelling current.

Nearly 90 cells have been tested with acetylcholine. About half of them were excited. This excitation had a characteristic long latency and persisted for many seconds after stopping the expelling current. Other choline esters, acetyl-β-methylcholine and carbamylcholine, also excited these cells. These cholinoceptive cells are chiefly concentrated in the superficial layers of the hippocampal cortex, between the alvear surface and 1 mm deep. Their receptors are principally muscarinic as atropine selectively blocked the excitatory response to acetylcholine but had no effect on that of L-glutamate, while dihydro-β-erythroidine was ineffective. However, with dimethyl-(+)-tubocurarine the acetylcholine response could occasionally be selectively depressed for many minutes. Further investigation is needed to determine whether acetylcholine is acting on pyramidal cells and has a genuine transmitter action at this site.

Most of the cells reacting to 5-hydroxytryptamine showed a progressive depression and some of them took up to 45 sec before they recovered their former excitability. A small number of cells were excited by 5-hydroxytryptamine.

The responses of hippocampal cells to these substances are qualitatively similar to those described for neocortical cells by Krnjević & Phillis (1963).

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Inhibition of noradrenaline uptake by drugs

SIR,—The *in vivo* studies of Axelrod, Whitby & Hertting (1961), Hertting, Axelrod & Whitby (1961), Hertting, Axelrod & Patrick (1962) and Axelrod, Hertting & Potter (1962) and the *in vitro* studies of Dengler, Spiegel & Titus (1961) showed that the uptake of noradrenaline into sympathetically innervated tissues can be inhibited by a wide variety of drugs. Among the compounds reported to act as inhibitors of noradrenaline uptake were cocaine, reserpine, guanethidine, imipramine, chlorpromazine and the adrenergic blocking agents dichloroisoprenaline (DCI), ergotamine and phenoxybenzamine. The present study was undertaken as a quantitative investigation of the potencies of these and certain other drugs as inhibitors of noradrenaline uptake.

In previous reports from this laboratory the kinetics of noradrenaline uptake in the isolated perfused rat heart and the inhibition of this process by a group of sympathomimetic amines have been described (Iversen, 1963; 1964). The methods used in the present experiments are described in these papers. Drugs were added to the perfusing medium, which also contained ^{14}C-noradrenaline. The uptake of noradrenaline was measured by analysing the ^{14}C-noradrenaline content of the heart at the end of a 10 min. perfusion. Each drug concentration was tested on a group of four hearts and the results were expressed as the mean percentage inhibition of noradrenaline uptake in the drug-treated group when compared with the uptake in drug-free controls. When sufficient data were available the drug concentration required to produce a 50% inhibition of noradrenaline uptake (ID50) was calculated.

TABLE I. THE INHIBITION OF NORADRENALINE UPTAKE BY DRUGS IN THE ISOLATED RAT HEART

Drug	Drug concentration (M)	% inhibition of noradrenaline uptake	Drug ID50 (M)
Desipramine	1×10^{-8}	43·5	
Desipramine	1×10^{-7}	81·0	$1{\cdot}3 \times 10^{-7}$
Desipramine	1×10^{-6}	92·5	
Imipramine	1×10^{-7}	45·5	
Imipramine	1×10^{-6}	75·0	$9{\cdot}0 \times 10^{-8}$
Bisdesipramine	1×10^{-7}	37·0	—
Reserpine	1×10^{-6}	73·0	—
Chlorpromazine	1×10^{-5}	88·5	—
Cocaine	1×10^{-7}	25·0	
Cocaine	1×10^{-6}	68·0	
Cocaine	1×10^{-5}	95·0	$3{\cdot}8 \times 10^{-7}$
Guanethidine	2×10^{-6}	41·0	
Guanethidine	2×10^{-5}	78·5	$3{\cdot}3 \times 10^{-6}$
Bretylium	2×10^{-6}	10·0	
Bretylium	2×10^{-5}	60·0	$1{\cdot}4 \times 10^{-5}$
Adrenergic blocking agents			
Phenoxybenzamine	1×10^{-5}	91·5	
DCI	2×10^{-6}	50·5	
Pronethalol	5×10^{-6}	36·0	
Phentolamine	1×10^{-5}	66·0	
Dibenamine	1×10^{-5}	34·0	
Monoamine oxidase inhibitors			
Tranylcypromine	1×10^{-5}	88·0	
Harmine	1×10^{-5}	79·5	
Phenelzine	1×10^{-5}	69·0	
Isocarboxazide	1×10^{-5}	14·5	
Nialamide	1×10^{-5}	3·5	
Pargyline	1×10^{-5}	nil	
Iproniazid	1×10^{-5}	nil	

The results obtained with 21 drugs are presented in Table 1. Cocaine and imipramine were very potent inhibitors of noradrenaline uptake. As reported by Titus & Spiegel (1962) the mono-*N*-methyl derivative of imipramine (desipramine) is an even more potent inhibitor of noradrenaline uptake than

imipramine. Desipramine is the most effective inhibitor of noradrenaline uptake so far described, it is approximately 30 times more potent than cocaine, 7 times more than imipramine and 6 times more than (–)-metaraminol (Iversen, 1964). The primary amine derivative of imipramine (bisdesipramine) was also tested but this compound proved to be less effective than desipramine.

The uptake of noradrenaline into tissues is thought to represent a major mechanism in the inactivation of noradrenaline. Drugs which act as inhibitors of this inactivation process should therefore have the property of potentiating the actions of noradrenaline on the smooth muscle receptor sites in sympathetically innervated tissues, since in the absence of tissue uptake more noradrenaline will be made available to interact with such receptor sites. This potentiation will include the effects of noradrenaline released from endogenous stores within the tissues by nerve impulses and the effects of noradrenaline introduced exogenously to the tissue. These potentiating effects are well known in cocaine and have also been reported in other inhibitors of uptake such as guanethidine and phenoxybenzamine (Stafford, 1963). The potent inhibitors of uptake, imipramine and desipramine are also particularly effective in potentiating the actions of noradrenaline (Sigg, Soffer & Gyermek, 1963). In agreement with this hypothesis, the present findings that desipramine is more potent than imipramine as an inhibitor of noradrenaline uptake agree with the pharmacological findings that desipramine is more effective than imipramine in potentiating the actions of noradrenaline.

With imipramine and desipramine it is possible that many of their pharmacological properties can be explained by their ability to prevent the inactivation of noradrenaline by tissue uptake. However, this is not so with most of the other compounds listed in Table 1. For example drugs whose major action is thought to be a blockade of either α- or β-adrenergic receptor sites were also found to act as inhibitors of noradrenaline uptake. Of the five adrenergic blocking agents tested all had some activity as inhibitors of uptake. In contrast to previous reports (Hertting & others, 1961; Lindmar & Muscholl, 1964) phentolamine and dibenamine were found to act as inhibitors of uptake, though these compounds were only weakly active. In the present experiments no correlation was apparent between the ability to block either α- or β-receptors and the ability to inhibit noradrenaline uptake, since both α- and β-blocking drugs were active as inhibitors of uptake. These results thus do not support the view that the inactivation of noradrenaline by tissue uptake involves an uptake or binding at either α- or β-adrenergic receptors (Kirpekar & Cervoni, 1963).

The present findings emphasise the importance of considering the possibility that a drug which affects adrenergic mechanisms may well have multiple sites of action. This point is also well illustrated by the results obtained when a group of monoamine oxidase inhibitors was tested. In addition to the ability to inhibit the enzyme monoamine oxidase, three of the seven drugs tested possessed the ability to inhibit noradrenaline uptake. Similarly guanethidine and bretylium were active as inhibitors of noradrenaline uptake, though it seems unlikely that this property can account for the adrenergic blockade produced by these compounds. In confirmation of the findings of Hertting, Axelrod & Patrick (1962) *in vivo*, bretylium was found to be approximately ten times less potent than guanethidine in inhibiting the uptake of noradrenaline.

Brodie & Beaven (1963) have proposed that the depleting action of reserpine on the tissue stores of noradrenaline can be explained by the inhibition of noradrenaline uptake produced by this drug. They suggested that reserpine inhibits an active accumulation of noradrenaline into adrenergic nerves, and

that in the absence of this uptake process the intracellular noradrenaline leaks out of the tissue by passive diffusional processes. The present results do not support this hypothesis, since there was no correlation between the ability to inhibit noradrenaline uptake and the ability to deplete the endogenous noradrenaline content of tissues among the compounds tested. Although reserpine and guanethidine are active as noradrenaline depleting agents and are also effective as inhibitors of uptake, there were several other equally potent inhibitors of noradrenaline uptake such as cocaine, imipramine and chlorpromazine, which are without effects on the levels of endogenous noradrenaline in tissues (Muscholl 1961; Gey & Pletscher, 1961).

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Letters to the Editor

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