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Histidine decarboxylase in experimental tumours

ROLF HÅKANSON

Histidine decarboxylase activity has been demonstrated in some experimental tumours by direct enzyme assay. The kinetic properties of semi-purified preparations of the histamine-forming enzyme from Rous rat sarcoma and Walker rat mammary carcinoma were similar to those of the "specific" histidine decarboxylase of the foetal rat. Transplants from a malignant hamster melanoma had no "specific" histidine decarboxylase but high dopa decarboxylase activity. The lack of histidine decarboxylase in this tumour seems to indicate that a high histamine-forming capacity is not indispensable for tumour growth.

A high rate of histamine biosynthesis has been demonstrated in some tissues characterized by rapid cell multiplication (cf. Kahlson, 1961); the liver of the foetal rat is a conspicuously rich source of histidine decarboxylase (Kahlson, Rosengren, Westling & White, 1958; Burkhalter 1962). Apart from neoplastic mast cells (Hagen, Weiner, Ono & Lee, 1960) certain experimental tumours are potent in forming histamine; Mackay, Marshall & Riley (1960) found a high histidine decarboxylase activity in a rat hepatoma and Håkanson (1961) demonstrated a considerable histamine-forming capacity in transplants from a rat mammary carcinoma, an observation which was later confirmed by Hallenbeck & Code (1962).

At least two enzymes are capable of catalysing the biosynthesis of histamine in mammalian tissues: the non-specific aromatic L-amino-acid decarboxylase (Udenfriend, Lovenberg & Weissbach, 1960; Lovenberg, Weissbach & Udenfriend, 1962)—also referred to as dopa decarboxylase (Rosengren, 1960)—and the more specific variety, which occurs in high amounts in tissues of the foetal rat (Ganrot, Rosengren & Rosengren, 1961; Burkhalter, 1962; Håkanson, 1963) and mouse (Håkanson, to be published). This enzyme has also been demonstrated in the bone marrow of the rat (Håkanson, 1964) and in the gastric mucosa of the rat (Håkanson & Owman, 1966) and mouse (Håkanson, to be published); in the latter species high activities of "specific" histidine decarboylase appear also in the renal cortex of pregnant animals (Håkanson, to be published, cf. Rosengren, 1963). The kinetic properties of histidine decarboxylase from the foetal rat have been the object of extensive investigation (Håkanson, 1963, 1966a).

Some data on the characteristics of the histamine-forming enzyme of rat hepatoma have been reported by Mackay, Riley & Shepherd (1961). The possibility of a connection between histamine formation and tumour growth (cf. Kahlson, 1961) prompted a more detailed study on the properties of histidine decarboxylase from some experimental non-mast-cell tumours.

Material and methods

The following experimental tumours were examined: Rous sarcoma of the rat (Ahlström & Jonsson, 1962), Walker rat mammary carcinoma

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256 (cf. Håkanson, 1961) and a malignant hamster melanoma (type M, Mel 1 of Fortner) described by Salamon & Storck (1961). Fresh tumour tissue (approx. 5 g) was collected and homogenized in 2 volumes of ice-cold 0·1 M phosphate buffer, pH 7·0. The particulate material was spun down (20,000 × *g*, 20 min, 0°) and discarded. The supernatant was used as enzyme source. In some experiments the enzyme was precipitated with ammonium sulphate as specified in 'Results'; the precipitate was taken up in buffer and dialysed overnight. Incubations were in an atmosphere of nitrogen at 37°. As a rule pyridoxal-5-phosphate (1 μg/ml) was added to the incubation medium which consisted of the enzyme extract diluted with 0·1 M phosphate buffer to a final volume of one ml. Dopa decarboxylase activity was determined at pH 7·0 by a radiometric method (Håkanson & Owman, 1965; Håkanson, 1966b); 3 μg ^{14}C-DL-dopa (5·75 mc/mM, Radiochemical Centre, Amersham) was added as substrate. Histidine decarboxylase activity was examined at various pH values and with various concentrations of substrate by fluorometric determination of the amount of histamine produced (Shore, Burkhalter & Cohn, 1959, cf. Håkanson, 1963); when a high sensitivity was needed a radiometric method was employed, in which 2 μg ^{14}C-L-histidine (32 mc/mM, Radiochemical Centre) was added as substrate (Håkanson, 1966b); the incubation was at pH 7. Specimens of each tumour were stained for the demonstration of mast cells by the histochemical technique of Bloom & Kelly (1960).

Results

Rous rat sarcoma. The tumour was found to contain about 10 μg histamine (free base) per gram fresh tissue. Only few mast cells occurred in the tumour. The tissue was devoid of dopa decarboxylase but a high histidine decarboxylase activity could be demonstrated (Table 1). The

TABLE 1. MAST CELLS, HISTAMINE CONTENT AND ENZYME ACTIVITIES OF SOME EXPERIMENTAL TUMOURS

Tumour	Mast cells	Histamine, μg/g	Dopa decarboxylase activity*	Histidine decarboxylase activity
Rous rat sarcoma	very few	9	0	10
Walker rat mammary carcinoma ..	absent	2·5	0	2
Malignant hamster melanoma ..	absent	0·6	32	0

* Enzyme activities were determined by radiometric micromethods (Håkanson, 1966b) and expressed as ng amine produced per 10 mg tissue in 1 hr.

histamine-forming enzyme was almost quantitatively recovered in the fraction precipitated with ammonium sulphate at between 25 and 40% saturation. The properties of this enzyme were very similar to those of histidine decarboxylase from the foetal rat: the optimum pH of the reaction changed with the substrate concentration (Fig. 1); the Michaelis-Menten constants, which were derived from Lineweaver-Burk (1934) plots of initial velocities, varied with pH (Fig. 2). Michaelis constants defined with respect to certain specific ionized species of histidine were fairly

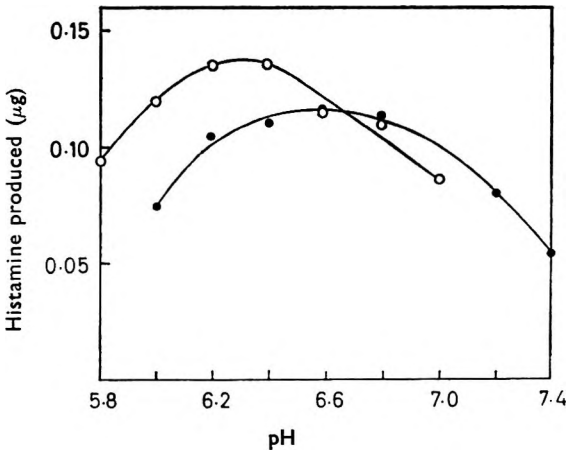


FIG. 1. Variation of the optimum pH of histidine decarboxylase from Rous sarcoma of the rat with the substrate concentration. Enzyme activity is expressed as μg histamine produced in 1 hr. The incubation medium was made up to a total volume of 1 ml with 0·1 M phosphate buffer. Incubations were under nitrogen at 37°. ○——○, 5×10^{-3} M histidine; pyridoxal-5-phosphate 1 μg/ml. ●——●, 5×10^{-4}M histidine; pyridoxal-5-phosphate 1μg/ml.

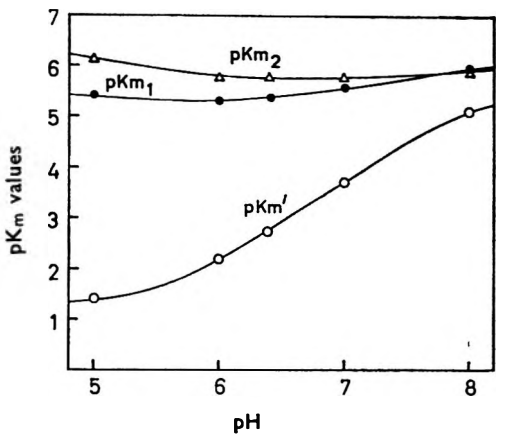


FIG. 2. Apparent pK_m (pK_m') (○——○) versus pH. The pK_{m_1} (●——●) and pK_{m_2} values (▲——▲) were calculated from equations 1 and 2 respectively.

constant within the experimental pH region (Fig. 2). These "true" Michaelis constants (K_m) were derived from the apparent Michaelis constants (K_m') at given pH by the equations:

$$K_m' = K_m\left(1 + \frac{(H^+)}{10^{-9}}\right) \quad .. \quad .. \quad .. \quad (1)$$

or

$$K_m' = K_m\left(1 + \frac{(H^+)}{10^{-6}} + \frac{(H^+)}{10^{-9}} + \frac{(H^+)^2}{10^{-15}}\right) \quad .. \quad .. \quad (2)$$

(The K values of histidine were approximated to 10^{-6} and 10^{-9}. The carboxyl-group was assumed to be ionized throughout). Equation 1 refers to the ionic form of histidine having an ionized carboxyl-group and a non-ionized α-amino-group (see appendix). The corresponding species in equation 2 is the one with ionized carboxyl-group and non-ionized imidazole- and α-amino-groups. A similar mathematical treatment of other species of histidine produced K_m values which varied widely within the experimental pH region. The consequences and implications of similar observations on the properties of foetal rat histidine decarboxylase have been discussed elsewhere (Håkanson, 1963, 1966a).

Walker rat mammary carcinosarcoma 256. The tumour had no dopa decarboxylase and considerably less histidine decarboxylase activity than the Rous sarcoma (Table 1). Mast cells were absent and the histamine content was low (cf. Håkanson, 1961). The histamine-forming enzyme of this tumour was similar to that of the Rous sarcoma; the apparent K_m changed with pH in an identical manner.

Hamster melanoma. This tumour had high dopa decarboxylase activity (cf. Håkanson, Möller & Stormby, 1965) but no histidine decarboxylase activity could be detected by any of the methods used (Table 1). The histamine content was low and mast cells were absent from the tumour tissue.

Discussion

The present results suggest that the Michaelis constant of histidine decarboxylase from some experimental tumours must be defined in terms of a specific ionized species of histidine, presumably the one with the ionized carboxyl group and non-ionized imidazole- and α-amino-groups (cf. Håkanson, 1963). Apparent K_m values (K_m values with respect to total amount of histidine present) were determined from Lineweaver-Burk (1934) plots over a wide range of pH values and were noted to decrease as the pH of the incubation medium was increased. Michaelis constants defined with respect to "true" substrate were derived from these apparent K_m values by relations 1 or 2 and were found to be fairly constant throughout the pH range examined; this observation seems to support the assumption that the enzyme attacks a certain ionic species of histidine. It appears that the species of histidine that predominates at the pH region where enzyme activity is optimal does not necessarily correspond to the species of histidine that constitutes the enzyme-substrate complex. A more detailed analysis of the kinetics of histamine formation, which considers also the ionization of the enzyme, will be published elsewhere (Håkanson, 1966a, and to be published).

The present results clearly show that the histamine-forming enzyme demonstrated in the Rous sarcoma and in the Walker rat mammary carcinoma is different from the aromatic L-amino-acid decarboxylase and very similar to the more specific variety of the histamine-forming isoenzymes.

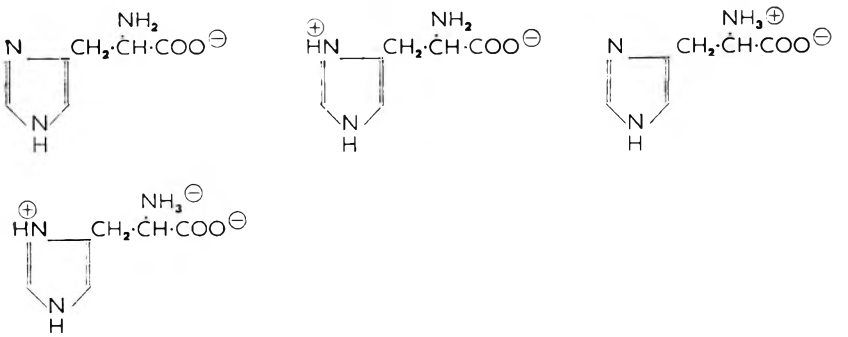
Kahlson has postulated a connection between a high rate of histamine

formation and rapid tissue growth. This view has been challenged by several authors (Kameswaran, Telford & West, 1961, Mackay, Reid & Shepherd, 1961; Kameswaran & West, 1962, Rosengren, 1966). An active biosynthesis of histamine may be a common phenomenon in experimental tumours of the rat and mouse but the lack of histidine decarboxylase in the hamster melanoma seems to indicate that a high histamine-forming capacity is not a necessary feature of tumour growth.

Acknowledgements. Supported by grants from Riksföreningen mot Cancer, The Royal Physiographic Society and the Medical Faculty of Lund. Experimental tumours were provided by Dr. N. G. Stormby, Department of Pathology, Lund, Sweden.

APPENDIX

Histidine occurs in various ionized species within the experimental pH region:



The corresponding conservation equation for histidine is

$$(S_t) = (S_1) + (S_2) + (S_3) + (S_4)$$

The concentration of each ionic variety of histidine is governed by the law of mass action and expressed by the following equations, which are derived from the conservation equation. (The K values are approximated to 10^{-6} and 10^{-9}.)

$$(S_1) = \frac{(S_t)}{1 + \frac{(H^+)}{10^{-6}} - \frac{(H^+)}{10^{-9}} - \frac{(H^+)^2}{10^{-15}}} \qquad (S_2) = \frac{(S_t)}{1 + \frac{10^{-6}}{(H^+)} - \frac{10^{-6}}{10^{-9}} + \frac{(H^+)}{10^{-9}}}$$

$$(S_3) = \frac{(S_t)}{1 + \frac{(H^+)}{10^{-6}} - \frac{10^{-9}}{10^{-6}} + \frac{10^{-9}}{(H^+)}} \qquad (S_4) = \frac{(S_t)}{1 + \frac{10^{-6}}{(H^+)} - \frac{10^{-9}}{(H^+)} + \frac{10^{-15}}{(H^+)^2}}$$

However, the enzyme may attack several ionized varieties of histidine indiscriminately. These are defined by the following relations: $(S_5) = (S_1) + (S_2)$, $(S_6) = (S_1) + (S_3)$, $(S_7) = (S_3) \quad (S_4)$, $(S_8) = (S_2) + (S_1)$.

The concentration of these ionic varieties are given by the following equations:

$$(S_5) = \frac{(S_t)}{1 + \frac{(H^+)}{10^{-9}}}; \qquad (S_6) = \frac{(S_t)}{1 + \frac{(H^+)}{10^{-6}}}; \qquad (S_7) = \frac{(S_t)}{1 + \frac{10^{-9}}{(H^+)}}; \qquad (S_8) = \frac{(S_t)}{1 + \frac{10^{-6}}{(H^+)}}$$

Equations (1) and (2) in the text refer to S_6 and S_1 respectively.

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The relation between noradrenaline content of rabbit heart muscle and the amount of k-strophanthin needed to produce arrhythmias

L. ANGELUCCI, G. LORENTZ AND MIRELLA BALDIERI

Possible relations between the sensitivity of the heart to the arrhythmia-inducing property of k-strophanthin and myocardial storage of noradrenaline were investigated. Sensitivity to the glycoside was increased in rabbits pretreated with reserpine in doses which depleted noradrenaline, but not in rabbits pretreated with tyramine in doses which are known to reduce noradrenaline content. Sensitivity to the glycoside was restored to normal by an infusion of noradrenaline not earlier than 72 hr after reserpine treatment, but not by an infusion given at 24 hr. Infusion of noradrenaline did not itself reduce the sensitivity to k-strophanthin in normal rabbits. The glycoside acutely reduced the myocardial store of adrenaline and noradrenaline in normal rabbits. The possibility that cardiac sensitivity to k-strophanthin is regulated by *bound* but not by *unbound* noradrenaline is discussed.

ACCORDING to Cairoli, Reilly, Ito & Roberts (1961) the effect of ouabain on myocardial behaviour is brought about through the liberation of noradrenaline. Tanz (1964) has expressed the view that "the presence of a significant concentration of catecholamines is necessary in order that the myocardium . . . be able to respond to exogenously administered cardiac glycosides. If this be true . . . then one might liken the normal role of cardiac catecholamines to a *permissive* action."

The possibility of a relation between heart sensitivity to k-strophanthin and myocardial concentration of noradrenaline has been investigated on the following premises: if the action of k-strophanthin on the heart in some way depends on the local stores of catecholamines it might be that (1) the glycoside does alter these stores, (2) their previous depletion modifies the cardiac response to k-strophanthin, (3) this response can be restored to normal by effecting a restoration of myocardial concentrations of noradrenaline.

Experimental

METHODS

Preliminary experiments were made to establish the reproducibility of a "sensitivity test" to k-strophanthin in the rabbit. The animal, maintained under light anaesthesia (20 mg/kg i.v. of sodium pentobarbitone and thereafter 0·025–0·050 mg/kg/min in a saline drip of 0·125–0·250 ml/min through the marginal vein of the right ear), was infused through the marginal vein of the left ear with 13·5 μg/min of k-strophanthin (in 0·16–0·20 ml of saline) until the appearance of electrocardiographic abnormalities. These generally consisted of bursts of ventricular extrasystoles or disordered atrioventricular conduction. This was taken as the end point, sensitivity being expressed in μg/kg of k-strophanthin received by the animal at the end point. When the same rabbit underwent a second test a similar degree of sensitivity to k-strophanthin was observed.

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The experiments were made in 45 rabbits of either sex (2–4 kg). After a first test the animals were grouped according to size and degree of sensitivity and k-strophanthin sensitivity was re-assessed (i) immediately after an infusion of noradrenaline, (ii) at different times after reserpine treatment, (iii) as in (ii) but with an infusion of noradrenaline given immediately before the test, (iv) immediately after treatment with tyramine, (v) as in (iii) except that a treatment with tyramine was interposed between the infusion of noradrenaline and the test.

Whenever possible the different treatments were made successively in the same animals.

Reserpine (Serpasil, Ciba) was given, 0·5 mg/kg i.p., each day for three days.

Infusion of noradrenaline, 250 μg/kg (20 μg/ml of saline with sodium metabisulphite 0·2%), was effected slowly to avoid arrhythmias due to high blood concentrations of the infused amine. For this reason the duration of infusion was usually not less than 5-6 hr in normal rabbits and 7-8 hr in reserpinised animals.

Tyramine hydrochloride was given to normal rabbits as four separate intramuscular injections of 20 mg/kg at one-hrly intervals, and to reserpinised animals as two doses of 20 mg/kg i.m., the first immediately after completion of the infusion of noradrenaline, and the second dose 30 min later. This was followed immediately by the k-strophanthin test.

An interval of at least ten days was left between consecutive tests to k-strophanthin sensitivity, and of at least 30 days between consecutive treatments with reserpine.

Electrocardiographic tracings were obtained using standard lead 2.

Adrenaline and noradrenaline contents of the ventricular myocardium, brain and whole adrenals were estimated by a procedure which incorporates modifications to the method of Lund (1949). This was preferred after a comparative evaluation with other methods (Angelucci, Ajello & Baldieri, 1963).

Tissues samples (half of the ventricles along a transverse section, half of the brain along a longitudinal section, one whole adrenal) were washed rapidly with cold saline, dried on filter paper, weighed and frozen at $-40°$. The frozen sample was homogenised in 0·4N perchloric acid, centrifuged at 30,000 *g* for 10–15 min at 5°, the supernatant, filtered through a Jena Glass G4 funnel if necessary, was transferred to a 50 ml beaker containing 12·5 mg of sodium metabisulphite and 200 mg of sodium edetate and made up to 25 ml with 0·4N perchloric acid. After mixing, prepared alumina (400 mg) was added and the contents were brought to and maintained for 5 min at pH 8·6 with continuous stirring and titrimetric control. Stirring was then stopped and the mixture left to settle (about 30 sec.) The supernatant was discarded by suction and the alumina, after rapid washing several times with cool distilled water with constant stirring using an L-shaped glass rod, was eluted twice with 2·25 ml of 0·2N acetic acid, with stirring for 10 min. The combined eluates were centrifuged at 30,000 *g* for 15 min at 5° and finally divided in two 2 ml portions; one sample was brought to pH 3 and the other to pH 6·5–7·0. Both samples were

oxidised with manganese dioxide, centrifuged, their supernatants each mixed with a solution of ascorbic acid 0·1% in 5N sodium hydroxide and the fluorescence read in a spectrophotofluorimeter at 400/540 mμ. From this reading the reading of the blank, obtained by shaking for 1 hr 250 times/min, was then subtracted. At the same time as the estimations in tissue extracts, recovery and standard estimations were made with 2 ml of solutions of adrenaline and noradrenaline (100 ng/ml) and mixtures of both amines. Concentrations of amines in the sample were calculated using the formula:

$$x = \frac{[fS\ pH\ 7 \cdot (fAd\ pH\ 3 : fAd\ pH\ 7)] - fS\ pH\ 3}{[fNor\ pH\ 7 \cdot (fAd\ pH\ 3 : fAd\ pH\ 7)] - fNor\ pH\ 3}$$

$$y = \frac{fS\ pH\ 3 - (x \cdot fNor\ pH\ 3)}{fAd\ pH\ 3}$$

where: x = noradrenaline concentration ng/ml in the sample; y = adrenaline concentration ng/ml in the sample; f = fluorescence reading; Ad = 2 ml standard solution of adrenaline (100 ng/ml); Nor = 2 ml standard solution of noradrenaline (100 ng/ml); S = sample 2 ml.

The method outlined affords a recovery of at least 90% and agrees in its limit of sensitivity of 0·5 ng/ml of final reaction mixture with the results obtained by Anton & Sayre (1962) using one of the most sensitive spectrophotofluorimetric methods.

To detect residual tissues amines in reserpinised animals a microprocedure was adopted. The whole heart or brain (respectively about 11 and 13 g in rabbits of average body weight of 3·5 kg) was extracted and the amount of alumina was reduced to 200 mg; elution was with 1·0 ml of 0·2 N acetic acid. 0·5 ml of the eluate was then oxidised at pH 7 and taken through the above procedure with proportionally reduced amounts of reagents. The final volume of the reaction mixture in the microcuvette was 0·82 ml. With this procedure it was possible to detect amounts of amines in excess of 1 ng/g of tissue, since the limit of sensitivity of the method is 0·5 ng/ml of final reaction mixture (the fluorescence reading of sample and its blank are respectively 18·5 and 10·0) No differential reading in this condition between sample and its blank indicates absence of appreciable quantities of amines. It is possible to recover from the brain homogenates of reserpinised animals 80% of an added 10 ng of noradrenaline [net fluorescence reading 24 (sample 54, blank 30)].

The statistical significance of the differences between results of the various treatments has been analysed with Student's *t*-test.

Results

SENSITIVITY TO K-STROPHANTHIN

Reproducibility of sensitivity. The quantity (μg/kg) of the infused glycoside in two successive tests in the same rabbit to elicit clearcut electrocardiographic abnormalities was remarkably constant (Table 1), even though in the different strains there were different degrees of sensitivity. These appeared to be correlated with the size of the adrenal

glands. Thus, the average sensitivity to k-strophanthin (in μg/kg) infused at a constant amount per min remained fairly constant for each group of four rabbits (adult animals, weight 2 kg), provided there were no large variations in body weight in successive tests. It was therefore possible to make comparisons among results obtained for different groups, provided they showed similar degrees of control sensitivity (first test), as well as among results obtained for the same group in different treatments, provided each group acted as its own control (first test).

TABLE 1. DEPENDENCE OF CARDIAC SENSITIVITY TO K-STROPHANTHIN IN RABBITS ON FREVIOUS TREATMENTS. Three successive tests were made on each group of four. Sensitivity was measured as μg/kg of the glycoside infused until appearance of arrhythmias on the ECG. Group A and B were infused with 13·5 and group C with 10·5 μg/min. Mean values ± s.e.

Group	First test			days of interval	Second test			days of interval	Third test		
	Weight kg	k-Strophanthin			Weight kg	k-Strophanthin			Weight kg	k-Strophanthin	
		µg/kg	total µg			µg/kg	total µg			µg/kg	total µg
A	(no treatment before test) 2·63 ±0·23	246 ±9	649 ±57	14–143	(no treatment before test) 2·86[1] ±0·55	253[1] ±12	721[1] ±108	10–111	(after infusion of noradrenaline[5]) 3·42[2] ±0·56	214[2] ±15	728[1] ±80
B	(no treatment before test) 2·31 ±0·20	271 ±24	628 ±54	10	(24 hr after reserpine[4] treatment) 2·50[1] ±0·28	166[3] ±17	415[3] ±44	30	(24 hr after reserpine[4] treatment, with noradrenaline[5] infusion) 2·60[1] ±0·34	152[3] ±21	395[3] ±81
C	(no treatment before test) 2·15 ±0·00	398 ±43	853 ±87	10	(72 hr after reserpine[4] treatment) 2·41[1] ±0·40	263[3] ±23	633[3] ±102	30	(72 hr after reserpine[4] treatment, with noradrenaline[5] infusion) 2·76[3] ±0·24	385[1] ±52	1057[2] ±90

[1] Not different from the first test, $P > 0.05$.
[2] Different from the first test, $0.05 > P > 0.01$.
[3] Different from the first test, $P < 0.01$.
[4] 0·5 mg/kg each day for 3 days.
[5] 250 µg/kg in about 6–7 hr.

In most rabbits of equal body weight, for the same dose of k-strophanthin, abnormalities at the end point of the test consisted of ventricular extrasystoles, but in some animals disorders of conduction prevailed.

Sensitivity to k-strophanthin in rabbits pretreated with reserpine. Treatment with depleting doses of reserpine (0·5 mg/kg i.p. each day for three days) increased heart sensitivity to k-strophanthin. Data in Table 1 show that after reserpine treatment there was a significant reduction in the amount of glycoside necessary to reach the end point. After treatment with reserpine there was not only a reduction of the mean dose/kg needed to reach the end point, but also a significant reduction of the mean total dose administered, since the same rabbits were used in each of the successive tests even though there was an increase of mean body weight of the animals between one test and the next.

Electrocardiographic tracings showed a pattern of abnormalities in reserpinised animals similar to that of the controls, though appearing at different dose levels of k-strophanthin.

RELATION BETWEEN NORADRENALINE AND K-STROPHANTHIN

Sensitivity to k-strophanthin after an infusion of noradrenaline in normal rabbits. Infusion of noradrenaline in normal rabbits before the k-strophanthin test increased heart sensitivity to the glycoside. A statistically significant difference ($0{\cdot}01 < P < 0{\cdot}05$) was found when comparing end point doses in μg/kg (Table 1).

Sensitivity to k-strophanthin after an infusion of noradrenaline in rabbits pretreated with reserpine. The infusion of noradrenaline in rabbits 72 hr after reserpine restored normal heart sensitivity to k-strophanthin. Data in Table 1 show that there was no significant difference between the dose of glycoside needed to reach the end point in controls and the dose in rabbits pretreated with reserpine when the test was preceeded by an infusion of noradrenaline.

The infusion of noradrenaline before the k-strophanthin test did not restore normal sensitivity when made within 24 hr after reserpine (Table 1).

Sensitivity to k-strophanthin after tyramine in normal and reserpinised rabbits. Tyramine is known to decrease catecholamine content of the myocardium so it might be expected to give results similar to those of reserpine. Treatment with tyramine in doses which effect a partial depletion did not modify sensitivity in normal rabbits nor in those infused with noradrenaline 72 hr after pretreatment with reserpine. The figures were: normal rabbits $249 \pm 10{\cdot}9$ and $291 \pm 31{\cdot}6$ μg/kg respectively without and with tyramine before the test to k-strophanthin; reserpinised rabbits infused with noradrenaline $281 \pm 24{\cdot}0$ and $263 \pm 19{\cdot}0$ μg/kg respectively without and with tyramine before the test to k-strophanthin.

THE RELATION BETWEEN CONCENTRATIONS OF NORADRENALINE AND HEART SENSITIVITY TO K-STROPHANTHIN

Table 2 shows that infusion of k-strophanthin until the appearance of electrocardiographic abnormalities reduced the content of noradrenaline in the myocardium. The degree of this reduction was relatively independent of the extent of the electrocardiographic abnormalities since in rabbits with similar degrees of reduction of noradrenaline, some showed only ventricular extrasystoles, some ventricular fibrillation and others died a few hours after the infusion. In rabbits so killed a substantial reduction of the noradrenaline content in the brain was found, while in rabbits which showed only clear cut but rapidly reversible electrocardiographic abnormalities the reduction in the brain was much less. In both groups a statistically significant difference from controls was found for the content of adrenaline in the adrenals.

The decrease of the noradrenaline content in myocardium and brain associated with k-strophanthin administration was not affected, as evidenced by data in Table 2, when the animals were previously infused with noradrenaline.

As data in Table 3 show, treatment with reserpine wholly depletes the stores of adrenaline and noradrenaline in myocardium, brain and whole adrenals. Repletion of normal stores did not occur in 24 hr from the last injection of reserpine; at 72 hr the process was beginning. An infusion of

TABLE 2. TISSUES CONCENTRATIONS OF ADRENALINE AND NORADRENALINE IN RABBITS INFUSED WITH K-STROPHANTHIN (13·5 μG/MIN) OR NORADRENALINE (250 μG/KG IN ABOUT 7–8 HR) OR WITH BOTH. Number of animals in parentheses.

Treatment	Myocardium ng/g		Brain ng/g		Adrenal gland ng/mg	
	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
Controls (4)	128±40	1816±283	127±51	343±89	785±60	172±36
30 min after infusion of noradrenaline (3)	379	2088				
k-Strophanthin: lethal doses (6)	170[1]	1183				
	103[1]	967	45	76	572	
	248[2]	718	31	35		
	192[2]	932	27	36	612	
	216[2]	979	22	110	592	
		1130[2]	32	54	645	
	M185±54[4]	984±163[5]	31±8·5[5]	62±31·4[5]	605±31·1[5]	77±32[5]
k-Strophanthin: test doses (4)	96±54[4]	982±139[5]		264±35[4]	634±51[5]	
k-Strophanthin: test doses after infusion of of noradrenaline (2)	145	714		205		
	62	649		209		
	M103	681		207		

[1] Died with ventricular fibrillation during the test.
[2] Died after the test (more than 3 hr).
[3] Died after the test (within 3 hr).
[4] Not different from controls, $P > 0.05$.
[5] Different from controls, $P < 0.01$.
M = mean.

noradrenaline 24 hr after reserpine pretreatment was able to partially replenish stores of adrenaline and noradrenaline in the heart and in the adrenal glands (Table 3). But this infusion, although producing a significant response, was not enough to restore normal sensitivity to k-strophanthin. However if the infusion of noradrenaline was given 72 hr

TABLE 3. TISSUES CONCENTRATIONS OF ADRENALINE AND NORADRENALINE IN RABBITS TESTED FOR CARDIAC SENSITIVITY TO K-STROPHANTHIN AFTER TREATMENT WITH RESERPINE FOLLOWED OR NOT FOLLOWED BY INFUSION OF NORADRENALINE. Number of animals in parentheses.

Treatment	Myocardium ng/g		Brain ng/g		Adrenal gland ng/mg	
	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
Controls (4)	128±40	1816±283	127±51	343±89	785±60	172±36
k-Strophanthin test[3] only (4)	96±54[4]	982±139[5]		264±33[4]	634±51[5]	77±32[5]
24 hr after reserpine[1] treatment. No test (2)	<1	<1	<1	<1	0·3	0·05
Infusion of noradrenaline[2] 24 hr after reserpine[1] treatment. No test (4)	<1	<1	<1	<1	0·6	0·4
Infusion of noradrenaline[2] 24 hr after reserpine[1] treatment. Test[3] (2)	112±54	107±37	5	3	139±—	15±9
72 hr after reserpine[1] treatment. No test (2)	66	22	8	5	5	0·8
	101	34	8	6	9	1
Infusion of noradrenaline[2] 72 hr after reserpine[1] treatment. Test[3] (4)	97±47[4]	1076±247[5]	23±13[5]	54±15[5]	332±115[6]	82±42[6]

[1] 0·5 mg/kg i.p. each day for 3 days; last injection 72 or 24 hr before test or killing.
[2] 250 μg/kg in about 7–8 hr.
[3] k-Strophanthin 13 μg/min i.v.
[4] Not different from controls, $P > 0.05$.
[5] Different from controls, $P < 0.01$.
[6] Different from controls, $0.05 > P > 0.01$.

after treatment with reserpine, the myocardial stores were restored to normal as well as the sensitivity to k-strophanthin.

It was not possible to ascertain the concentration of noradrenaline in the myocardium after the infusion since the animals were then tested for sensitivity to k-strophanthin which, on its own, decreased the normal myocardial content of adrenaline and noradrenaline.

Discussion

Our experiments confirm already reported evidence that k-strophanthin decreases myocardial noradrenaline (Cession-Fossion, 1962). Depletion of catecholamine stores by reserpine resulted in an increase in heart sensitivity to the glycoside-induced arrhythmias. Repletion of myocardial stores of noradrenaline in reserpinised rabbits by an infusion of this amine restored normal sensitivity to the production of arrhythmias by k-strophanthin.

Since the increase above the normal of the noradrenaline content in the myocardium, which can be obtained by infusing the amine into normal rabbits, was not associated with a decrease of heart sensitivity to k-strophanthin, it could be argued that this sensitivity depends on the absolute value of the concentration of the amine.

TABLE 4. CARDIAC SENSITIVITY TO K-STROPHANTHIN AND MYOCARDIAL CONCENTRATIONS OF ADRENALINE AND NORADRENALINE IN RABBITS. SENSITIVITY WAS MEASURED AS μG/KG OF THE GLYCOSIDE GIVEN UNTIL APPEARANCE OF ARRHYTHMIAS ON THE ECG RECORD. Number of animals in parentheses.

Treatment	k-Strophanthin[1] test μg/kg	Myocardium ng/g	
		Adrenaline	Noradrenaline
Group 1: Controls (4)	—	128 ± 40	1816 ± 283
Group 2: test only (4)	305 ± 36	96 ± 54[5]	982 ± 139[5]
Group 3: 72 hr after reserpine[2] treatment (2)	—	83	28
Group 4: First test (4)	271 ± 24	—	—
Second test at 24 hr from reserpine[2] treatment	166 ± 17[5]	—	—
Third test, preceded by infusion of noradrenaline,[3] at 24 hr from reserpine[2] treatment	152 ± 21[5]	< 1	< 1
Group 5: First test (4)	398 ± 43		
Second test at 72 hr from reserpine[2] treatment	263 ± 23[5]		
Third test, preceded by infusion of noradrenaline[3] at 72 hr from reserpine[2] treatment	385 ± 52[4]	97 ± 46[4]	1076 ± 247[5]

[1] 10·5 μg/min for groups 2 and 5; 13·5 μg/min for group 4.
[2] 0·5 mg/kg i.p. each day for 3 days; last injection 72 or 24 hr before test or killing.
[3] 250 μg/kg in about 7–8 hr.
[4] Not different from controls or first test, $P > 0.05$.
[5] Different from controls of first test, $P < 0.01$.

In Table 4 the degree of heart sensitivity to k-strophanthin is compared with the concentrations of noradrenaline in the myocardium in different experimental situations: it appears that for sensitivity to remain at normal values, a minimal local concentration of noradrenaline is needed which is larger than that found 72 hr after reserpine treatment, when increased sensitivity still persists. This minimal concentration can be built up by an appropriate suitably timed infusion of noradrenaline.

After reserpine depletion the immediate repletion of the myocardial store of *unbound* noradrenaline is still possible (Kirpekar & Furchgott, 1964). Repletion of the store of *bound* noradrenaline—by far the larger part of the amine store in the heart—remains inhibited for some days at least (Bhagat & Shideman, 1964). Since an infusion of noradrenaline can restore the concentration of the amine to normal only if given at a critical time after reserpine treatment (72 hr), the substantial increase in concentration of the *bound* form of noradrenaline would appear to be the phenomenon with which recovery of normal heart sensitivity to k-strophanthin is associated.

In favour of the hypothesis that *bound* noradrenaline is involved in the regulation of heart sensitivity to k-strophanthin is the fact that we found tyramine, in doses which partially reduce the *unbound* concentration of noradrenaline in the myocardium, did not interfere with heart sensitivity to the glycoside both in normal rabbits and in rabbits infused with noradrenaline 72 hr after reserpine treatment. Doses of tyramine which maximally deplete the myocardial amines, still leave enough noradrenaline to maintain an optimal functional transmission (Chidsey, Harrison & Braunwald, 1962); moreover the infusion of noradrenaline in the dog 24 hr after reserpine restored cardiac responsiveness to tyramine but not to sympathetic stimulation (Gaffney, Chidsey & Braunwald, 1963). It is this part of total myocardial catecholamine, not depleted by tyramine and not replaceable by an infusion of noradrenaline in the first hours after reserpine, which may also be associated with the sensitivity of the heart to the arrhythmia-inducing property of k-strophanthin.

During the preparation of this paper we learned of the increased toxicity of k-strophanthin to guinea-pig heart-lung preparations 24 hr after reserpine (Carpi & Oliviero, 1965).

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Coating of pharmaceutical tablets: the spray-pan method

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A new method of coating tablets uses the revolving coating pan, but the coating materials are applied in solution or suspension, as a spray from a centrifugal disc atomiser. Addition of coating materials is uniform, controlled, and at a rate which allows even deposition on the tablet. Dry powders need not be used and the process is therefore dust-free. The products of the method compare favourably with tablets coated commercially and with tablets coated by the standard method in laboratory size (16 inch) pans when assessed by uniformity of weight tests, roundness measurement, and radiographic examination.

THE standard method of coating tablets in revolving pans involves addition of powder and syrups alternately, or of suspensions, in varying amounts with drying between each addition. The volumes and manner of addition, and the method of drying yield lumpy surfaces in the early stages of coating and, although this may quickly change the shape to that of a rough oblate spheroid it requires smoothing syrup coats to give an acceptable product. It would facilitate the study of coating and the achievement of a uniform product if coats of any desired thickness could be built up from the commencement of the coating by deposition of smooth layers.

In the present work this has been achieved by applying the coating materials in the form of sprayed droplets and a spray-pan method suitable for laboratory-scale pan coating, and amenable to scale-up is described. The standard coating pan is used to support the tablet bed and the coating materials are sprayed onto the revolving tablets in a controlled manner from a centrifugal disc atomiser.

Materials and methods

SPRAYING APPARATUS

The apparatus is shown diagrammatically in Fig. 1. The spinning disc (4) may be of metal or plastic and is smooth and circular; the laboratory model is 2 inches in diameter and 0·2 inch thick; it is driven anticlockwise by a variable speed motor (Desoutter M 10 x[1]). The speed of the disc was determined (±1%) by a stroboscopic tachometer (Stroboflash 1200E[2]). The disc is located centrally in a rectangular stainless steel or brass shield (5) open towards the coating pan, with an aperture (7) of variable width to allow spraying of fluids with different spray characteristics. The size of this aperture, the position of the disc relative to the pan, and its distance from the tablets are regulated to allow the spray to fall only on the rolling tablets in a line from front to back of the pan. The bottom of the shield is suitably designed to facilitate drainage (80% of spray) which is returned to

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[1] Desoutter Brothers Ltd., The Hyde, Hendon, London, N.W.9.

[2] Dawe Instruments Ltd., Western Avenue, Acton, London, W.3.

the thermostatically controlled feed reservoir (1) by means of a pump (8).[3] Coating materials are fed onto the centre of the spinning disc through an intermediate glass reservoir (3) which eliminates the pulsatile nature of the flow from the glandless metering pump (2).[4] The metering pump controls the feed rate in conjunction with the size of the nozzle on the intermediate reservoir. In this laboratory study the spraying equipment was used together with a 16 inch bench type coating pan (6),[5] revolving at 30 rpm and receiving 15–20 ft^3/min air supply at 60°.

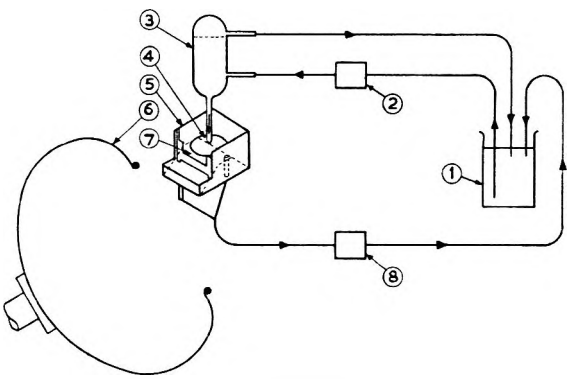

FIG. 1. Flow diagram for spray-pan coating process. 1. Feed reservoir. 2. Metering pump. 3. Intermediate reservoir. 4. Spinning disc. 5. Stainless steel shield. 6. Coating pan. 7. Aperture. 8. Pump.

SPRAY CHARACTERISTICS

Paraffin-coated microscope slides were passed through the spray and the adhering droplets were sized microscopically to yield D_{av} the average drop diameter, and D_{sv} the volume-surface mean drop size (Friedman, Gluckert & Marshall, 1952) which can also be thought of as the diameter of a drop whose volume to area ratio would be the same as that for the entire spray (Adler & Marshall, 1951).

TABLETS

A mixture of icing sugar and lactose, equal parts, was granulated with about 90 ml gelatin solution (20% w/v in water) per kg of sugar-lactose mixture and compressed on a Manesty B3 rotary machine using deep-concave punches 5/16 inch 13/32 inch and ½ inch diameter giving average tablet weights of 0·16, 0·38 and 0·60 g, respectively.

COATING MATERIALS

All fluids were applied at 60° to previously warmed tablets (except polishing solution which was used at room temperature). All applications

[3] Multifix Peristaltic Pump, Fisons Scientific Apparatus Ltd., Loughborough, Leics.

[4] Watson-Marlow Flow Inducer type MHRE, Watson-Marlow Air Pump Co., Marlow, Bucks.

[5] Stainless steel pan, BCP 2, Manesty Machines Ltd., Liverpool.

were made to tumbling tablets without blown air. Coating stages are given in Table 1.

TABLE 1 COATING MATERIALS AND STAGES

Batch number	Coating Stages						
	1	2	3	4	5	6	7
II, III	Acacia syrup suspension A		Syrup		—		—
IV	Titanium dioxide syrup				Syrup		Polishing solution
V	Kaolin syrup				Syrup		Polishing solution
VI	Calcium carbonate syrup				Syrup		Polishing solution
VIII, IX	Syrup				—		—
XI, XII	Gelatin syrup				—		—
IVa, XIa, XIIa	Gelatin syrup and kaolin				Titanium dioxide syrup	Syrup	—
XIIb,	Gelatin syrup and kaolin		Titanium dioxide syrup	Syrup	Polishing solution	—	—
Ia	Gelatin syrup and kaolin	Titanium dioxide syrup	Syrup	Polishing solution	—	—	—
Suspension coating	Titanium dioxide syrup				Syrup		—

Syrup was syrup B.P.; gelatin syrup was syrup containing 1·3% w/w gelatin [170 bloom (Cox)]. Titanium dioxide syrup, calcium carbonate syrup, kaolin syrup and barium sulphate syrup each contained 13·3% w/w of the appropriate solid in gelatin syrup; titanium dioxide was titanium dioxide AE (British Titan Products); the other powders were B.P. quality. Acacia syrup contained 1 g acacia B.P., 10 g sucrose and 10 ml water. Acacia syrup suspension A contained 1 g acacia, 4 g titanium dioxide, 10 g sucrose and 10 ml water; acacia syrup suspension B was similar except that it contained half-quantity titanium dioxide. Polishing solution was 2% w/v white beeswax in carbon tetrachloride. The pan load was always 3 kg (uncoated weight).

SPRAY-PAN COATING METHOD

For a disc speed of 8,000 rpm and feed-rate of 100–150 ml/min the following operation cycle was used: spray 1–1½ min; tumble only, 1 min without air; blown hot air 1–2 min with tumbling. This cycle was repeated until the desired coated weight was attained. Using this method, tablets could be coated with syrup or gelatin syrup alone; subcoating and dusting powder were not used. Tablets were also coated using titanium dioxide syrup, calcium carbonate syrup, kaolin syrup, barium sulphate syrup or acacia syrup suspension A or B and finished with syrup; dusting powder was not applied.

Tablet coated weight was double the uncoated weight. When suspensions were used, two-thirds of the weight of coat was supplied by the suspension, syrup contributing the remainder.

STANDARD COATING METHOD

(a) *Sub-coating.* Gelatin syrup or acacia syrup, 70–100 ml per application, was added alternately with kaolin or titanium dioxide, 250–300 g per application, to a predetermined weight. Tablets were allowed to roll 1–2 min between liquid and powder applications and for 2–3 min after addition of powder, followed by hot air blown in for about 10 min. (b) *Smoothing.* Acacia syrup suspension or one of the powder syrups, 50–100 ml per application, was followed by tumbling (2 min) without blown air, then hot air (5 min) until smoothing was complete. (c) *Finishing.* Syrup, 20–30 ml per application, was used together with tumbling (2 min) without air, and hot air (5 min) to final weight. Syrup at room temperature, with tumbling and no air, was used for about the last four applications. (d) *Polishing.* Polishing solution (100 ml in one application) was added to the tablets in a clean pan and they were left to tumble 6 hr without air application.

SUSPENSION COATING

Titanium dioxide syrup, or acacia suspension B, 50–100 ml per application, was applied as in the smoothing operation of the standard coating method, to a predetermined coat weight; finishing and polishing were as in the standard method.

COATED TABLETS OF COMMERCE

Samples of coated tablets were obtained from commercial sources together with samples of the same batches taken at different stages in the coating process. Although formulations and methods were not disclosed the stages of coating provided have been named arbitrarily according to appearance of the tablets, thus stage 1 represents sealing; stage 2, sub-coating; stages 3–5 include smoothing, colouring and finishing.

UNIFORMITY

Weight. Uniformity of weight was tested in the uncoated tablets and after each 0·1 g increase in coat weight during the coating processes. For the test, 20 tablets were weighed individually, and the average weight, standard deviation and coefficient of variation were calculated.

Coating thickness. Uniformity of thickness at various stages of coating was tested by means of the Talyrond 2[6] which measures roundness. The measurements were made on the circumferential edge of the tablet together with a reference computer which provides an integrated reference circle, from which departures from roundness are measured to allow calculation of the integrated average departures from the reference circle, designated the mean line average. The graph obtained for each tablet shows the

[6] The Rank Organisation, Rank Taylor Hobson Division, Leicester.

differences between the circumferential edge and a true circle at the magnification used (× 100). The graph is, therefore, not a pictorial representation of the tablet. The roundness of the core tablets was also measured.

Radiography of coated tablets. Tablets were also radiographed at the various stages of coating.

Results and discussion

The major difficulties in the standard method of pan coating of tablets arise in the method of coat build-up, especially in the sub-coating stage. In laboratory size (16 inch) pans it is difficult to prevent the early occurrence of frank lumpiness which then requires excessive coating material to smooth out the coat. In large pans (36 inch to 7 ft) which are usually rotated at speeds similar to the laboratory-size pan, the grinding action of the weight of the tablet bed (about 30 to 200 kg respectively) and the more efficient mixing and better drying conditions on the greater tablet area exposed, all tend to prevent lumpiness. The difficulties of the standard method were greatly reduced in the 16 inch pan by suspension coating but difficulty in obtaining controlled, uniform addition of coating material remained.

The 16 inch pan is therefore limited in development work using the standard and suspension methods, and results are difficult to scale-up faithfully. Also, this size pan is not entirely satisfactory for research in coating which, at the present time, is concerned not only with depositing certain types of coating but also with the accurate construction of coatings which will provide reproducible rates of release of drugs incorporated in the coat. In large-scale technique, using the standard method, the subjective element, which attends the timing and amount of coating materials added is less than satisfactory for routine production of precisely constructed coats, where reproducibility between, and uniformity within, batches is important. Nevertheless, the revolving pan of the traditional method provides a suitable support for the tablet bed during the application of coatings, and large weights (200 kg) can thus be coated without the necessity of starting with undesirably hard core tablets to minimise the damage which can occur in fluidised beds (Singiser, Heiser & Prillig, 1965).

The revolving pan has been retained in the present spray-pan method, but the means of addition of coating materials has been made more objective by a centrifugal disc atomiser which permits uniform, controlled addition of coating materials at a known rate, without the hand mixing frequently used in the traditional method. Spraying may, of course, be accomplished by pressure, twin-fluid or rotary atomisers. The centrifugal disc is the simplest type of atomiser; it is easily used and cleaned, high pressures are not required, and it can be used with fluids of widely varying properties over a wide range of feed rates. This gives greater possibility of controlled variation in application conditions and of producing sprays of uniform droplet size.

Droplet character from the centrifugal disc type of rotary atomiser depends on the disc (speed, diameter, energy transmission to the liquid

surface), the liquid (flow characteristics, surface tension, density), and on the atmosphere through which the spray passes (Adler & Marshall, 1951; Friedman & others, 1952; Fraser & Eisenklam, 1956; Fraser, Eisenklam & Dombrowski, 1957).

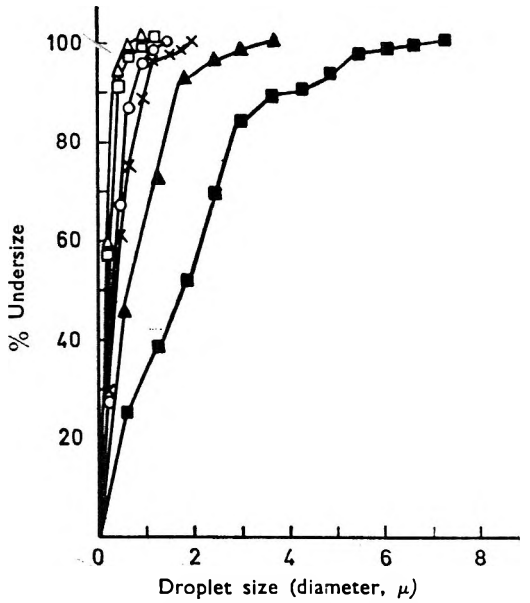

FIG. 2. Cumulative undersize distribution for sprayed coating suspension at a feed rate of 100 ml/min and several disc speeds. Disc speeds (rpm): ■ 2,000, ▲ 4,000, × 6,000, ○ 8000, □ 10,000, △ 18,000.

To obtain uniform drop size, the centrifugal force should be large compared with the gravitational force, disc rotation should be vibrationless, liquid feed rates should be uniform, and disc surface should be smooth (Walton & Prewett, 1949; Adler & Marshall, 1951). Sufficient centrifugal force was obtained by spraying at a disc speed of 8000 rpm or greater; below this, uniformity of drop size was generally unsatisfactory (Fig. 2). Vibrationless rotation was approached by using an air driven rotor; uniformity of flow rate was achieved by using the metering pump-reservoir combination; the Perspex disc provided a smooth surface.

SPRAY CHARACTERISTICS

Fig. 3 shows the variations in D_{sv} with disc speed. D_{sv} was chosen to characterise the spray because it is related to the amount of surface created, and creation of new surface is the aim of atomisation. Increasing the disc speed creates more coating fluid surface thus facilitating even spreading and drying on the surface of the tumbling tablets. Increase in feed rate increases D_{sv}, hence greater feed rates require faster disc speeds for constant D_{sv}, although the difference between 100 ml/min and 200 ml/min is small (Fig. 3). Figs 2 and 3 indicate that, under the conditions

of the experiment, 8000 rpm was the lowest speed for reasonable uniformity of spray; speeds greater than this did not greatly decrease D_{sv}. For increasing disc speed or decreasing feed rate, the mechanism of droplet formation changes from ligament or film formation to direct drop formation which is known to produce sprays with uniform droplets (Walton & Prewett, 1949; Hinze & Milborn, 1950; Adler & Marshall, 1951; Friedman & others, 1952; Fraser & Eisenklam, 1956; Fraser & others, 1957).

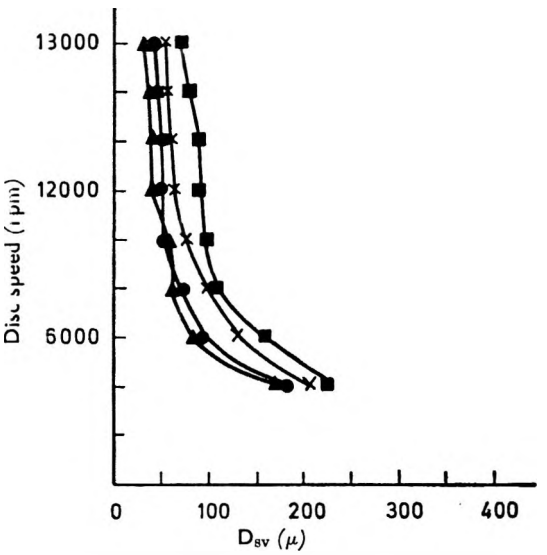

FIG. 3. Variation in volume-surface mean droplet size (D_{sv}) with disc speed for two feed rates of a coating suspension (titanium dioxide syrup) and a coating solution (syrup). ▲ Syrup, 100 ml/min, ● Titanium dioxide syrup, 100 ml/min, ■ Syrup, 200 ml/min, × Titanium dioxide syrup, 200 ml/min.

SPRAY-PAN METHOD

The adjustable aperture (Fig. 1) in the apparatus, while conserving spray, allows determination of the width of horizontal spray falling on the tablets and is, therefore, an additional control of the amount, as well as the position, of the spray. The spraying cycle must be determined for the conditions of the experiment but once determined it is reproducible in inexperienced hands and capable of automatic control. Scale-up is straightforward, whereas in the standard coating method large scale coating is more readily accomplished than laboratory scale coating. The process is also dust-free, and a complete coat can be built up using the spray-pan method without adding any free powder. This is because the uniform addition of liquid in spray form allows a rapid drying rate without sticking or lump formation and the use of suspensions allows rapid coat build-up.

The reasonable constancy of coefficient of variation in weight as the coat builds up in the spray-pan method, contrasts with the picture of coefficient of variation not only for the standard method in the 16 inch pan, but also

for commercial tablets which have been coated in larger pans (Fig. 4D). Figs 4A, 4B and 4C show comparisons between the plots of coefficient of variation versus coating stage for tablets of different core size and show that for a range of core sizes of frequent use the relationship is the same. Batches IVa, XIa and XIIa, coated by the standard method, yield a higher level of coefficient of variation of weight throughout the process, whereas batches II–VI, VIII, IX, XI, XII (spray-pan method) consistently have a coefficient of variation less than 4%, and close to that of the cores.

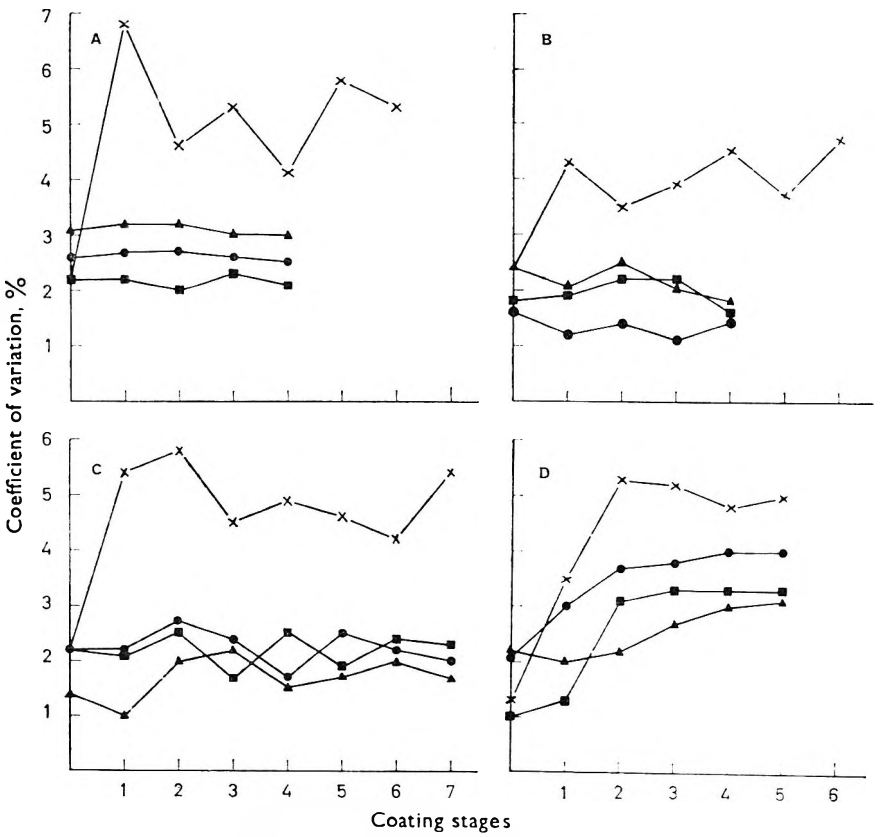

FIG. 4 A–D. Coefficient of variation of weight of tablets at various stages in coating. Batches II-VI, VIII, IX, XI, XII, spray-pan coated; IVa, XIa, XIIa coated by standard method. Other details in Table 1. Diameter of core tablets: A. 5/16 inch; B. 13/32 inch; C. 1/2 inch; D. Commercial, various core diameters. Symbols: A. X = Batch XIIa; ▲ = Batch II; ● = Batch III; ■ = Batch XII. B. X = Batch XIa; ▲ = Batch XI; ■ = Batch VIII; ● = Batch IX. C. X = Batch IVa; ● = Batch IV; ▲ = Batch V; ■ = Batch VI. D. X = Commercial Tablets (1); ● = Commercial Tablets (2); ▲ = Commercial Tablets (3); ■ = Commercial Tablets (4).

It is interesting to note that the limit set by the Swedish Pharmacopoeia (1946) of a coefficient of variation of 4·5% for uncoated tablets, is not exceeded even by tablets after coating, when this is done by the spray-pan

method; indeed, tablets coated by this method are markedly less variable in weight throughout the process of spray-pan coating (Figs 4A–C).

Turning to commercial tablets (Fig. 4D), the effect of subcoating (apparently by a standard method) on the coefficient of weight variation can be clearly seen in the increase occurring in the early stages. Whether the standard method of coating is in 16 inch pans or pans of greater size, it is clear (Figs 4A–D) that the variation in weight distribution during the early (subcoating) stages determines the variation in subsequent stages.

Roundness measurement, performed on the circumferential edge of the tablet by means of the Talyrond, was preferred to surface texture measurement (B.S. 1134: 1961). Examples of Talyrond graphs are in Fig. 5.

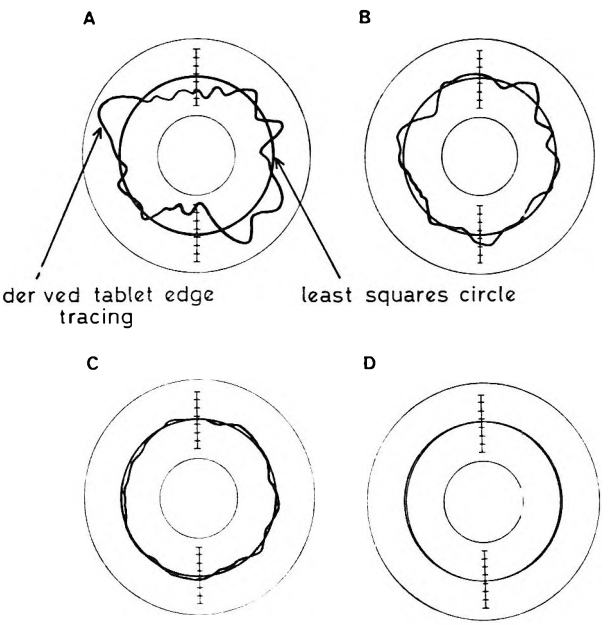

FIG. 5 A—D. Examples of Talyrond graphs. Each figure shows the least squares circle together with the tracing, derived from the tablet edge, which describes the differences between that edge and the least squares circle; superimposed scale (normally 12 such scales with subdivisions appear; these have been reduced for clarity). A. Batch XIa. B. Commercial tablet. C. Batch XI. D. Core.

The roundness measurements made at the various stages of coating are plotted in Fig. 6. They support the results of the uniformity of weight measurements and the findings from radiography (which proved to be a suitable means of visualising coating layers) and show that the spray-pan coated tablets are rounder (smoother) at all stages of coating than the tablets coated by the standard method either in the 16 inch pan or commercially. Fig. 6 shows that the early coats in the coating procedure influence the ease with which a smooth final coat can be obtained. When the mean line average (see p. 792) for the early coats is high, it remains high

throughout the later coating stages, although some commercial tablets did show a slight irregular fall in mean line average in the late coating stages indicating that the smoothing coats were fulfilling, to some extent, the textbook function. High mean line averages after subcoating are important in view of the empirical teaching and practice of the tablet coaters regarding subcoating, any inelegance of which is commonly thought to be readily and completely concealed by the succeeding coats. In the 16 inch pan it was possible to obtain a consistently decreasing mean line average in the later coating stages by decreasing the amount of subcoating (compare batches XIIa, XIIb, Fig. 7). A mean line average value (at the end of the coating) similar to that for suspension coating and spray-pan coating (batch Ia, Fig. 7) was made possible by using only 0·1 g subcoating.

Comparison of roundness measurements with the uniformity of weight results shows that, in the standard coating method, particularly when used on a laboratory scale (Fig. 4A–C batches IVa, XIa, XIIa) but also noticeably on a commercial scale (Fig. 4D samples 1, 2, 3, 4), both departure from roundness and coefficient of variation of weight increase steeply in the

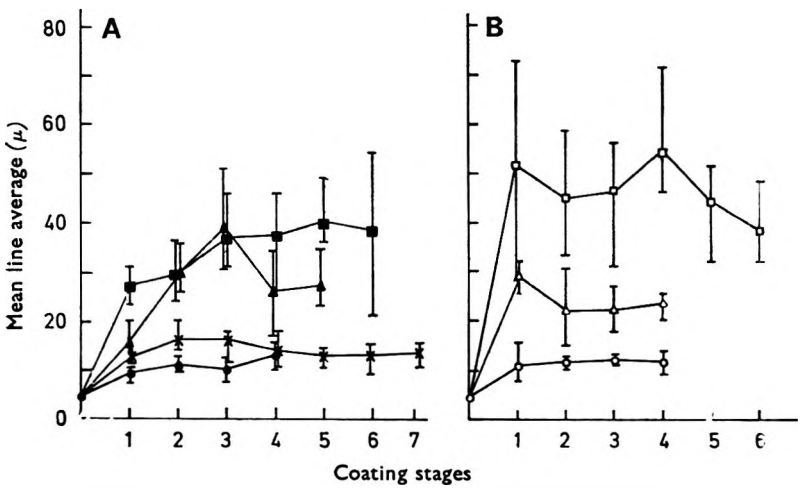

FIG. 6 A—B. Variation in mean line average (uniformity of thickness) as coating proceeds. Each point is the mean of five readings. A. ■ = batch XIIa, ▲ = commercial 1, × = batch IV, ● = batch XII. B. □ = batch XIa, △ = commercial 5, ○ = batch XI.

early stages of coating, the subsequent "smoothing" coats failing to restore the low values for both types of measurement seen in the core tablets. However, in the spray-pan method the coefficient of variation of weight remains constant throughout the process in spite of the small initial increase in departure from roundness of from 3μ (mean line average) for the core, to 10–15μ for the later stages.

This difference between the two methods of coating is caused by the use of free powder in the subcoating stage in the standard method for which

there is, in many instances, little justification, since the need is better met by using a liquid suspension applied preferably in atomised form.

Other attempts to improve on the pan-coating of tablets have been restricted to automation of the stages in the method (Clay & D'Angelo, 1956; Rieckmann, 1963; Steinberg, 1964) or to the method itself (Lachman, 1966) but the properties of the resulting coat were not described. Kwan (1961) used an automated process to study drying of coatings; Butensky (1961), also using an automated process, confirmed experimentally the experience of tablet coaters that it is the powder-liquid additions and

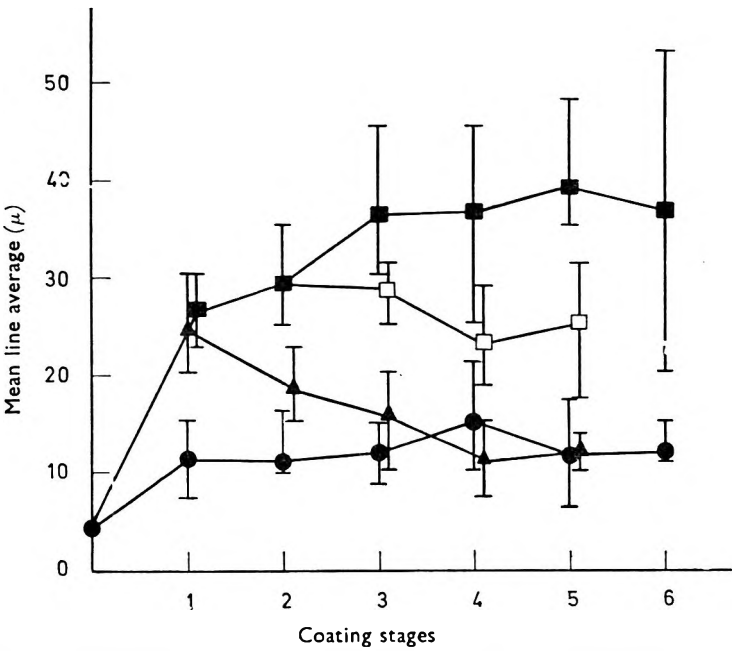

FIG. 7. Variation in mean line average (uniformity of thickness) as coating proceeds. Each point is the mean of five readings. ■ = Batch XIIa. ■ → □ = Batch XIIb. ▲ = Batch Ia. ● = suspension coating (Batches XIIa and XIIb followed a common path to coating stage 2, where the batch was divided; the two batches were thereafter treated separately as shown in Table 1).

ratios, coupled with drying rates, which lead to the difficulties of the standard method. Butensky (1961), who restricted his study to the subcoating stage, found that, within this stage of tablet coating, the coefficient of variation of tablet weight rose to about 7%, this maximum occurring before the final subcoating application. Although our coefficient of variation is lower, this is in general agreement with our findings for the standard method.

The difficulty in achieving uniformity has been stressed by Mattocks (1958), and this difficulty has hindered development of research in tablet coating. Spray-pan coating provides a method capable of the degree of

standardisation necessary to allow its use in the study of many of the problems in tablet coating which remain.

Acknowledgements. We thank the following: Messrs. G. Cochrane and I. Aird for help in building the spraying apparatus; Mr. T. S. Wylie of the Natural Philosophy Department of this University for help in the X-ray studies; Mr. C. A. Scoles and Mr. R. Kirk of the National Engineering Laboratory, East Kilbride, for helpful discussions and use of Talyrond 2; Mr. L. Sweeney for technical assistance; the companies of the British, American and Japanese pharmaceutical industries who co-operated generously in supplying samples of tablets at various stages of coating.

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The effects of acetates of aliphatic alcohols on the cholinergic nerve structures and the acetylcholine receptor of the guinea-pig ileum

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Isoamyl acetate, n-butyl acetate, n-amyl acetate and n-propyl acetate produced contractions of the isolated ileum of the guinea-pig. These were inhibited by atropine, procaine and by cooling. These acetates and also s-butyl, t-butyl and n-octyl acetate behaved as inhibitors of acetylcholine at 14 ± 1°. The acetylcholine content in the organ bath fluid increased after 60 min incubation of the ileum with isoamyl acetate. The results indicated that the agonistic acetates produced contraction of ileum through the liberation of acetylcholine from the cholinergic nerve-endings. All the acetates behaved as inhibitors of acetylcholine when they combined with the acetylcholine receptor on the muscle.

IT was recently reported (Takagi, Takayanagi, Ishida & Moritoki, 1965, Takagi & Takayanagi, 1966) that two mechanisms could be responsible for the acetylcholine liberation from the cholinergic nerve plexus of the guinea-pig ileum. One was liberation by 5-hydroxytryptamine and picric acid (Takagi & Takayanagi, 1962, 1965) and was blocked by morphine and strychnine; the other was effected by phenyl acetate and was resistant to the action of morphine and strychnine. We have now examined the modes of action of acetates of aliphatic alcohols on the isolated ileum of the guinea-pig.

Methods and materials

The experiments were made on 3 to 4 cm strips of male guinea-pig ileum suspended in Tyrode solution, gassed with oxygen 95% and carbon dioxide 5%. The responses of the gut were recorded on a smoked paper. The bath of 40 ml capacity was usually maintained at 32°. In some experiments the temperature of the bath fluid was lowered to 14 ± 1° for 1 to 1½ hr. The fundamental methods of the agonist-antagonist techniques are in principle the same as those reported by Brownlee and his colleagues (Harry, 1962; Brownlee & Johnson, 1963).

Some experiments were made on the frog rectus abdominis muscle to test the effects of isoamyl acetate on acetylcholine-induced contractions.

The acetylcholine released from the isolated ileum was estimated by the method of Schaumann (1957). Ilea (20 g) from three guinea-pigs were cut into pieces 1 to 1·5 cm in length which were mixed and divided into two lots (each of 10 g). Tyrode solution (40 ml) which contained physostigmine salicylate (3×10^{-5} M) was added to one lot which was then incubated at 32° for 1 hr, during which isoamyl acetate (2×10^{-3} M) was added at zero time and then after 15, 30 and 45 min incubation (total 8×10^{-3} M). The control group was similarly treated except that Tyrode was added instead of isoamyl acetate. After centrifuging the

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incubation mixture, the acetylcholine content of the supernatant was assayed on the excised rectus abdominis muscle of the frog treated with physostigmine salicylate (3×10^{-5} M). Results are the means of at least 8 experiments.

DRUGS

Drugs under test. n-Amyl acetate, isoamyl acetate, n-butyl acetate, s-butyl acetate, t-butyl acetate, n-octyl acetate, n-propyl acetate.

Agonists. Acetylcholine chloride, dimethylaminoethyl acetate hydrochloride, 5-hydroxytryptamine creatinine sulphate, nicotine bitartrate, phenyl acetate, picric acid.

Antagonists. Atropine sulphate, morphine hydrochloride, procaine hydrochloride.

Results

EXPERIMENTS ON THE ISOLATED ILEUM

Isoamyl, n-butyl, n-propyl and n-amyl acetate contracted the isolated ileum, but n-butyl, n-propyl and n-amyl acetate were only partial agonists (Table 1). The contractions were inhibited by treatment for 5 min with

TABLE 1. INTRINSIC ACTIVITIES (i.a.) AND AFFINITIES (pD_2 OR pA_2) OF ACETATES OF ALIPHATIC ALCOHOLS

	i.a.	pD_2	pA_2 (ACh)	pA_2 (IA)	pA_2 (ACh)	pD_2
Temperature (°C)	32	32	32	32	14 ± 1	14 ± 1
n-Octyl acetate	0	—	3·65	3·58	3·76	—
n-Amyl acetate	0·38	2·82	2·99	2·89	3·07	—
Isoamyl acetate (IA)	0·92	3·52	—	—	2·60	—
n-Butyl acetate	0·62	3·20	2·56	2·66	2·81	—
s-Butyl acetate	0·1	—	3·00	2·93	2·98	—
t-Butyl acetate	0	—	2·81	2·78	2·83	—
n-Propyl acetate	0·32	2·91	3·24	3·23	3·19	—
Isopropyl acetate	0	—	3·20	3·08	3·19	—
Acetylcholine (ACh)	1·00	7·12	—	—	—	7·22
Dimethylaminoethyl acetate	1·00	5·25	—	—	—	5·26
Atropine	0	—	8·32	8·28	8·20	—

The pA_2 values were obtained as the competitive inhibitory activities against the agonist in the parentheses. i.a. = maximum response by test agonist/maximum response by acetylcholine.

atropine (2×10^{-8} M), for 60 min with procaine (2×10^{-4} M), or by cooling the ileum to 14 ± 1° for 1 to 1½ hr (Fig. 1) but not by 3 min treatment with morphine (10^{-5} M). Cooling and treatment with procaine also abolished the responses induced by 5-hydroxytryptamine (5-HT), picric acid, nicotine and phenyl acetate which have been shown to release acetylcholine from the cholinergic nerve plexus in the ileum, but the responses to acetylcholine were unaffected by this treatment (Fig. 1). The acetates which did not contract the ileum displaced the dose-response curves of acetylcholine and isoamyl acetate towards higher concentrations in a parallel manner indicating a competitive inhibitory action against acetylcholine and isoamyl acetate. Synergism between t-butyl acetate or n-propyl acetate and atropine was examined. The percent maximum

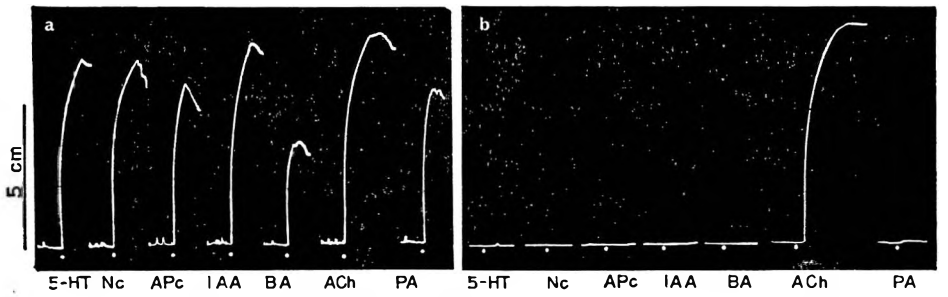

FIG. 1. Effect of cooling guinea-pig ileum on the responses produced by 5-hydroxytryptamine (5-HT), picric acid (PA), nicotine (Nc), phenyl acetate (APc), isoamyl acetate (IAA), n-butyl acetate (BA) and acetylcholine (ACh). (a) responses at 32°, (b) responses at 14 ± 1°. 5-HT, $3{\cdot}0 \times 10^{-5}$ M; Nc, $5{\cdot}0 \times 10^{-5}$ M; APc, $3{\cdot}0 \times 10^{-4}$ M; IAA, $1{\cdot}0 \times 10^{-3}$ M; BA, $2{\cdot}0 \times 10^{-3}$ M; ACh, $1{\cdot}0 \times 10^{-7}$ M; PA, $3{\cdot}0 \times 10^{-4}$ M. Note that only the response produced by acetylcholine was not inhibited by cooling.

contractions of the ileum to acetylcholine plotted against the molar concentrations of acetylcholine (log scale) are given in Fig. 2. Curve a is the control dose-response curve to acetylcholine, curve b is the dose-response curve to acetylcholine in the presence of t-butyl acetate ($1{\cdot}7 \times 10^{-3}$ M); curve c is the dose-response curve in the presence of atropine (2×10^{-8} M); curve d is the dose-response curve to acetylcholine in the presence of atropine (2×10^{-8} M) and t-butyl acetate ($1{\cdot}9 \times 10^{-3}$ M). It can be seen from Fig. 2 that the distance between curves a (acetyl-

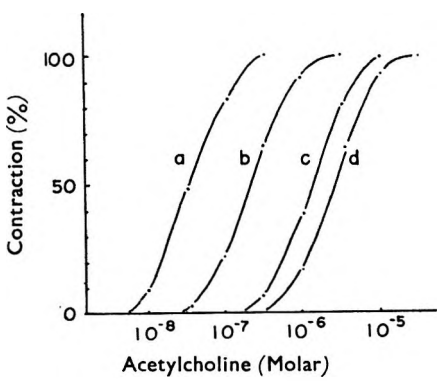

FIG. 2. Synergism between t-butyl acetate and atropine. (a) acetylcholine alone, (b) acetylcholine with t-butyl acetate $1{\cdot}7 \times 10^{-3}$ M, (c) acetylcholine with atropine 2×10^{-8} M, (d) acetylcholine with atropine and t-butyl acetate $1{\cdot}7 \times 10^{-3}$ M.

choline alone) and b (acetylcholine with t-butyl acetate, $1{\cdot}7 \times 10^{-3}$ M) was less than that between curves c (acetylcholine with atropine, 2×10^{-8}M) and d (acetylcholine with atropine, 2×10^{-8} M and t-butyl acetate, $1{\cdot}7 \times 10^{-3}$ M). Moreover, curve d was quantitatively identical to the

theoretical value calculated from equation 1, an equation for additive antagonism (Takagi & Takayanagi, 1964).

$$\frac{y}{y'} = \frac{A/K_A}{1 + A/K_A + B/K_B + C/K_C} \quad .. \quad .. \quad (1)$$

where: y' = maximum response, y = response induced by agonist A in the presence of antagonists B and C, and K_A, K_B, K_C are dissociation constants of the corresponding drugs (A, B, C).

According to Takagi & Takayanagi (1964), for potentiation between t-butyl acetate and atropine to occur the distance between the curves a and b must be equal to that between the curves c and d. The present result indicated that the synergism between t-butyl acetate and atropine was additive synergism and not potentiation. A similar result was obtained with atropine and n-propyl acetate. These results suggested that the inhibitory acetates combined with the acetylcholine receptors to inhibit the contraction due to acetylcholine. The effects of cooling the ileum on the actions of acetylcholine and dimethylaminoethyl acetate, both of which combine directly with the acetylcholine receptor to contract the muscle were further examined. The affinities of the agonists were expressed as the pD_2 values (negative logarithm of the concentration which produced 50% of the maximal contraction of muscle); the competitive inhibitory activities were expressed as the pA_2 values which were calculated from shift of a dose-response curve of an agonist using the table of van Rossum (1963). The pA_2 value of atropine against acetylcholine and the pD_2 values of acetylcholine and dimethylaminoethyl acetate were little affected by cooling the ileum to $14 \pm 1°$, so using acetylcholine as an agonist, the pA_2 values of the acetates tested at a temperature of $14 \pm 1°$ must represent their affinity for the acetylcholine receptor on the smooth muscle. The intrinsic activities and affinities (pD_2 or pA_2) of all the acetates of aliphatic alcohols tested on the isolated ileum of the guinea-pig are summarised in Table 1. These results suggest that the agonistic acetates, especially isoamyl acetate and n-butyl acetate, contract the ileum through the liberation of acetylcholine from its cholinergic innervation and that when these acetates combine with the acetylcholine receptor, they behave as competitive inhibitors of acetylcholine.

INCREASE OF THE OUTPUTS OF ACETYLCHOLINE FROM THE GUINEA-PIG ILEUM

The mean (± s.e.) of the spontaneous outputs of acetylcholine from the ileum (control group) was 3·2 ± 0·8 μg/g tissue/hr; the mean (± s.e.) of the amount of acetylcholine liberated from the ileum (test group) treated with isoamyl acetate (8×10^{-3} M) was 6·3 ± 1·2 μg/g tissue/hr. Thus isoamyl acetate accelerated the release of acetylcholine from the ileum.

EXPERIMENTS ON THE FROG RECTUS ABDOMINIS MUSCLE

Isoamyl acetate in concentrations up to 4×10^{-3} M did not contract the frog rectus abdominis muscle. After incubation of the muscle with isoamyl acetate (4×10^{-3} M) for 40 min, the affinity (pD_2) of acetylcholine

was not altered. This suggests that isoamyl acetate has only weak anticholinesterase activity, if any.

Discussion

Burgen (1965) has recently reported that 3,3-dimethylbutyl acetate and isoamyl acetate induced contraction of the guinea-pig ileum. On the other hand Takagi, Takayanagi & Fujie (1958) reported that isoamyl acetate did not contract the longitudinal muscle of the mouse ileum or the frog rectus abdominis muscle but behaved as a competitive and non-competitive antagonist of acetylcholine. This leads us to inquire into the possibility that the contractions of the guinea-pig ileum caused by isoamyl acetate might result from the activation of a receptor mechanism absent in the frog rectus. We have found evidence in favour of this possibility since the experiments described above have revealed that isoamyl acetate causes an increase in the acetylcholine output from the guinea-pig ileum. The fact that the effects of acetates of aliphatic alcohols, especially isoamyl acetate and n-butyl acetate were inhibited by atropine and procaine and by cooling, suggests that the site of action of these agonistic acetates must be in the nerve structures.

Two mechanisms have been postulated by Takagi & Takayanagi (1966) and Takagi, Takayanagi, Ishida & Moritoki (1965) for the liberation of acetylcholine from the guinea-pig ileum: the first mechanism is activated by electrical stimulation at low frequencies and by 5-HT and picric acid and is depressed by morphine; the second is activated by higher frequencies of electrical stimulation and by the action of phenyl acetate and is resistant to the inhibitory action of morphine. In the present experiments the responses induced by the agonistic acetates were not inhibited by 3 min treatment with morphine, so that acetylcholine released by them may result from the activation of the second mechanism.

It was recently reported by Johnson (1964) and Brownlee & Johnson (1965) that 5-HT and dimethylphenylpiperazinium activated different nerve pathways in the cholinergic nerve plexus of the guinea-pig ileum. Takagi, Takayanagi, Irikura & others (1965) have observed that spontaneous movements of ileum removed from guinea-pigs exhibiting morphine withdrawal symptoms were greatly increased and this increase of the spontaneous activity could be inhibited by morphine but not by hexamethonium. From this and from the further observations on the ileum from guinea-pigs exhibiting withdrawal symptoms that the effects of 5-HT were also greatly increased but those of nicotine were not altered, Takagi, Takayanagi, Irikura & others (1965) postulated that the increase of the spontaneous movements might be explained by the excitation of a nerve pathway activated by 5-HT but not by the nerve pathway activated by nicotine. This observation supports Johnson's conclusion on the nerve pathways.

The two nerve pathways proposed by Johnson (1964) would seem to constitute our acetylcholine liberation mechanism which is depressed by morphine.

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Complexation of penicillins and penicilloic acids by cupric ion

W. A. CRESSMAN*, E. T. SUGITA, J. T. DOLUISIO AND P. J. NIEBERGALL

A kinetic method was developed to study the interactions between cupric ion and penicillins G and V. Complexation was found to occur with the intact penicillins, followed by rapid hydrolysis of the complex into the corresponding penicilloic acid-cupric ion complex. The logarithmic stability constants for the interaction between cupric ion and penicillins G and V were found to be 2·61 and 2·24, respectively. In addition, the method of continuous variation was used to determine the logarithmic stability constants for the cupric ion-penicilloic acid complexes. These were found to be 4·20 and 4·50, for penicilloic V and G acids respectively.

UNDER mildly acid conditions penicillins G and V have been shown to be catalytically hydrolysed by cupric ion, into the corresponding penicilloic acids, at rates much too rapid for possible intact penicillin-cupric ion interactions to be measured by standard complexation techniques (Niebergall, Hussar, Cressman, Sugita & Doluisio, 1966). Therefore, a kinetic method was developed for determining the association constants for these interactions. Since the penicilloic acids were found to be the only degradation products produced under the conditions used, it also appeared desirable to determine their interactions with cupric ion. This was done spectrophotometrically using the method of continuous variation.

Experimental

REAGENTS AND EQUIPMENT

All the chemicals used were of reagent grade. The penicillin V (1530 units/mg) was supplied by Wyeth Laboratories, Inc. and the penicillin G (1595 units/mg) by the Eli Lilly Co. All solutions were made in water that had been deionised after distillation. In all work involving potentiometric titrations, the water was subsequently degassed by boiling for 30 min.

Titrations were done automatically using the Radiometer TTT-1c titrator with the Radiometer Titragraph model SBR2c. Spectral curves were obtained using a Beckman model DB spectrophotometer with a Photovolt model 43 linear-log recorder. Single wavelength determinations were made using an Hitachi-Perkin Elmer spectrophotometer.

Penicilloic acid solutions were made by the hydrolysis of penicillins at pH 12 for 15 min at room temperature in the manner described by Rapson & Bird (1963). The penicilloic acid solutions were adjusted to pH 6·50 and either used or discarded within 4 hr of preparation.

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COMPLEXATION BETWEEN INTACT PENICILLINS AND CUPRIC ION

Theory. One plausible explanation for the catalytic effect of cupric ion on the hydrolysis of penicillins to the corresponding penicilloic acids would be the rapid formation of a cupric ion-penicillin complex, which could then decompose rapidly into a penicilloic acid-cupric ion complex with the release of a proton. This would be indicated by:

$$P + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Y \xrightarrow{k_3} B + H^+$$

in which P is the intact penicillin, C represents cupric ion, Y represents the penicillin-cupric ion complex and B represents the penicilloic acid-cupric ion complex. In subsequent discussions, the above symbols enclosed in brackets will indicate molar concentration. The appearance of product may be given by:

$$d[B]/dt = k_3 [Y] \quad .. \quad .. \quad .. \quad .. \quad (1)$$

If the equilibrium is established much more rapidly than the degradation of the complex into products, and the penicillin concentration is much greater than that of the cupric ion, so that it remains essentially constant, the equilibrium constant, K, may be given by:

$$K = \frac{[Y]}{P_0 [C]} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

in which P_0 represents the initial penicillin concentration. Insertion of equation 2 into equation 1 results in:

$$d[B]/dt = k_3 K P_0 [C] \quad .. \quad .. \quad .. \quad (3)$$

The molar concentration of cupric ion at any time is:

$$[C] = C_0 - [Y] - [B] \quad .. \quad .. \quad .. \quad (4)$$

in which C_0 represents the initial cupric ion concentration. Using equation 2 to replace [Y] in equation 4 and rearranging yields:

$$[C] = \frac{C_0 - [B]}{1 + KP_0} \quad .. \quad .. \quad .. \quad .. \quad (5)$$

Equation 5 can be inserted into equation 3 to give:

$$d[B]/dt = \frac{k_3 K P_0}{1 + K P_0} (C_0 - [B]) \quad .. \quad .. \quad (6)$$

Integration of equation 6 under the condition that at time zero, B = O;

$$\log (C_0 - [B]) = \log C_0 - \frac{k_3 K P_0 t}{2{\cdot}303 + 2{\cdot}303\, K P_0} \quad .. \quad (7)$$

Thus, the appearance of product should be first order with a slope, A, given by:

$$A = \frac{k_3 K P_0}{2{\cdot}303 + 2{\cdot}303\, K P_0} \quad .. \quad .. \quad .. \quad (8)$$

This equation can be inverted to give:

$$1/A = \frac{2{\cdot}303}{k_3 K P_0} + \frac{2{\cdot}303}{k_3} \quad .. \quad .. \quad .. \quad (9)$$

The first order rate constant for the appearance of product is thus obtained for a number of solutions in which C_0 is kept constant, but P_0 is varied, always keeping P_0 much greater than C_0. A plot of $1/A$ versus $1/P_0$ should give a straight line with a slope equal to $2{\cdot}303/k_3$ K and an intercept equal to $2{\cdot}303/k_3$. Dividing the intercept by the slope would give the value for K.

Kinetic data. In the model proposed for the degradation process, equimolar amounts of hydronium ion and penicilloic acid-cupric ion complex would be produced. The proton release was therefore used to follow the kinetics of the reaction by the standard pH stat technique using solutions containing 1×10^{-4}M cupric chloride and penicillin concentrations ranging from 10 to 80×10^{-4}M at pH 5·50. The ionic strength was kept constant at 0·01 with potassium chloride. The reaction vessel used had a glass jacket which permitted the temperature to be kept at 30·00 ± 0·02°. The titrant was 0·0016N sodium hydroxide. The reaction vessel was continuously overlaid with a blanket of nitrogen throughout each determination. At the end of several determinations the solution was assayed for penicilloic acid content using the following modification of the method described by Pan (1954).

To 2 ml of a 0·20 M pH 5·50 acetate buffer containing 6×10^{-4}M cupric chloride add 4 ml of the solution to be assayed. The final concentration of penicilloic acid in the resulting 6 ml should not exceed 2×10^{-4}M. Add 1 ml of the Pan reagent and allow 200 sec for the colour to develop. Add 10 ml of distilled water and read the absorbance of the solution at 800 mμ in a 1 cm cell in exactly 100 sec.

In all instances in which solutions were assayed at the end of a kinetic run, the penicilloic acid content was within 3% of the theoretical 1×10^{-4}M. This was determined by comparing the absorbance of these solutions with that of solutions prepared to represent the reaction mixture at 100% reaction, i.e. 1×10^{-4}M cupric chloride and penicilloic acids, and an appropriate amount of penicillin, which had been simultaneously assayed using the modified Pan method. Finally a number of reaction mixtures were scanned through the ultraviolet region to determine the possible presence of penicillenic acids which absorb strongly at 322 mμ. The results of these scans verified our previous findings that penicillenic acids are not produced under these conditions.

To eliminate the non-catalysed degradation of the penicillins as a source of proton, several determinations were made using penicillin solutions (80×10^{-4}M), in the absence of cupric ion, but keeping the other conditions identical. Less than 2% of the penicillin G and none of the penicillin V degraded via the non-cupric ion catalysed route during the time interval used in this work.

PENICILLOIC ACID-CUPRIC ION INTERACTIONS

The method of continuous variations as modified by Woldbye (1955) was used. Beer-Lambert plots were obtained separately at 273 and 266 mμ for penicilloic V acid and cupric chloride, and at 245 mμ for penicilloic G acid and cupric chloride. These and all other determinations

were made in 0·034M acetate buffer at pH 5·50 at 30°. Equimolar stock solutions (1×10^{-3}M) of the penicilloic acids and of the cupric chloride were made. A series of solutions was prepared by adding (X)(V) ml of the penicilloic acid stock solution and (1-X)(V) ml of the cupric chloride stock solution to 30 ml of the acetate buffer. The final constant volume of these solutions is represented by V, and X is the fraction of the penicilloic acid stock solution. The difference between the absorbance of these solutions and the theoretical absorbance calculated from the Beer-Lambert plots was plotted against X conventionally.

The stability constants for 1 : 1 complexes were calculated by preparing solutions containing (X)(V) ml of a penicilloic acid stock solution and (1-X)(V) ml of a cupric chloride stock solution of a concentration different from that of the penicilloic acid stock solution. The solutions were added to 30 ml of the acetate buffer solution to give a total volume of 50 ml. The absorbance of each of these solutions was obtained at 273 mμ for penicilloic V acid and at 245 mμ for penicilloic G acid and then plotted against X. The value of X obtained at the maximum absorbance, X_{max}, was used in the following equation:

$$K = \frac{(r - 1)(1 - 2X_{max})}{(Cu^{++})(X_{max} + rX_{max} - 1)^2} \quad .. \quad .. \quad (10)$$

in which r is the ratio of penicilloic acid to cupric chloride in the stock solutions and the other terms have the meaning previously given. Two determinations were made using r values of 1·10 and 1·25 and a final chloride concentration of $4{\cdot}00 \times 10^{-4}$M.

Results

PENICILLIN-CUPRIC ION INTERACTIONS

The data in Fig. 1 demonstrate the first order dependency of the reaction, and the effect of P_0 upon the rate. In all instances the plots gave excellent results through more than three half lives. The rate constants, determined by least square methods, were used to determine the association constants according to equation 9. Fig. 2 shows representative plots of equation 9, indicating the validity of this method. The logarithmic association constants obtained in triplicate on penicillin G and penicillin V were 2·61 $\pm$ 0·01 and 2·24 $\pm$ 0·09 respectively.

PENICILLOIC ACID-CUPRIC ION INTERACTIONS

The spectral data for the method of continuous variation are shown in Figs 3 and 4, representing penicilloic G acid and penicilloic V acid, respectively. In both instances the lines intersect at X = 0·5, indicating a 1 : 1 complex, and justifying the use of equation 10 to obtain the association constants. The values for the logarithmic association constants from equation 10 were 4·20 $\pm$ 0·50 for penicilloic V acid and 4·50 $\pm$ 0·02 for penicilloic G acid.

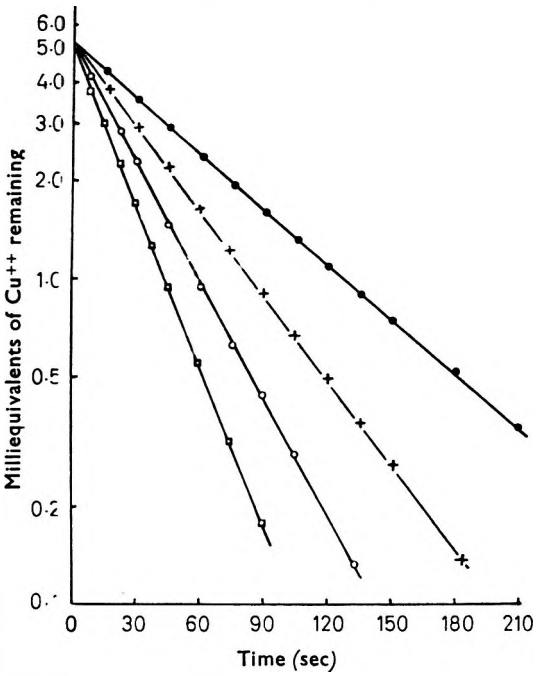


FIG. 1. First order dependency for the reaction between cupric chloride, 1×10^{-4}M, and penicillin V, -●-●- 10×10^{-4}M, -X-X- 16×10^{-4}M, -○-○- 30×10^{-4}M, and -□-□- 40×10^{-4}M, at pH 5·50, using 0·0016N sodium hydroxide as the titrant.

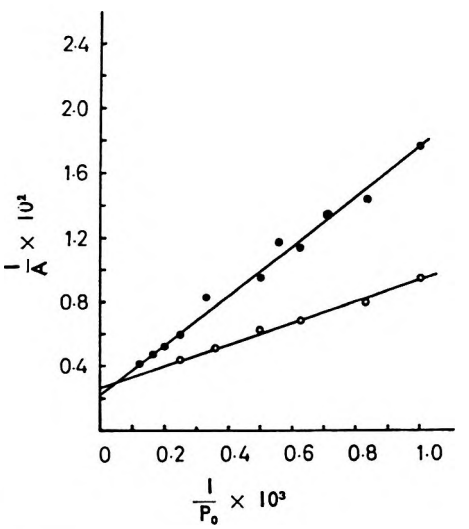


FIG. 2. Reciprocal of the apparent first order rate constant versus the reciprocal of the initial -●-●- penicillin V or -○-○- penicillin G concentrations, in solutions initially containing cupric chloride 1×10^{-4}M, at pH 5·50. Penicillin concentrations ranged from 10 to 80 $\times 10^{-4}$M.

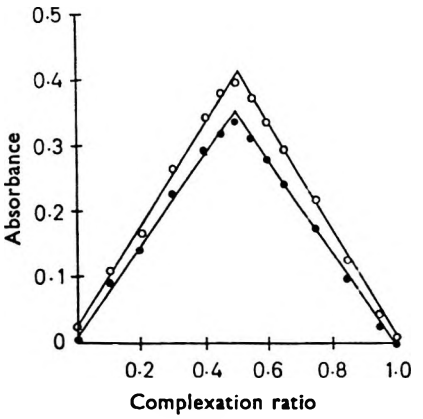


FIG. 3. Method of continuous variation to determine the complexation ratio between cupric ion and penicilloic V acid at 30° in 0·034M acetate buffer solutions at pH 5·50. Absorbance was recorded in 1 cm cells at —●—● 273 mμ and —○—○— 266 mμ.

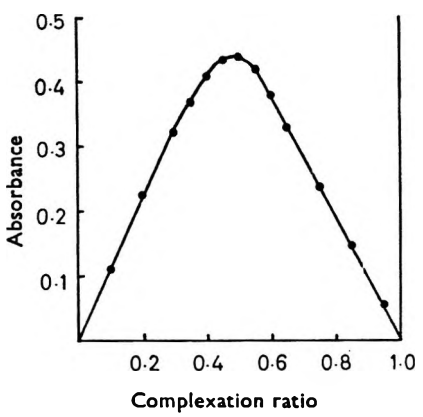


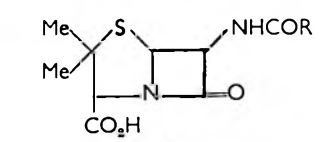
FIG. 4. Method of continuous variation to determine the complexation ratio between cupric ion and penicilloic G acid at 30° in 0·034M acetate buffer solutions at pH 5·50. Absorbance was recorded at 245 mμ using 1 cm cells.

Discussion

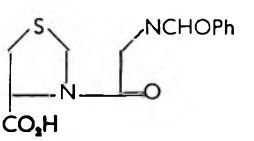
Adherence to first order kinetics through more than three half-lives, and the subsequent plots of the rate constants according to the proposed mechanism indicate the validity of the method for obtaining the association constants in question. Since the model and assumptions involved have been used successfully for years to obtain values for enzyme-substrate interactions, it would have been surprising indeed if the data did not fit. The major difference in our mechanism is a different balance equation for cupric ion, which unlike enzyme catalysts, is consumed in the reaction. The mechanism, however, cannot be firmly established solely on the basis of fit to complexation studies. Further kinetic analysis of this system is presently being undertaken.

The rate constants obtained indicate that the half lives for the cupric ion catalysed hydrolysis of penicillins V and G in unbuffered solutions at a pH 5·50 and ionic strength of 0·01, although varying with penicillin concentration, are approximately 50–100 sec. Thus any attempt to measure penicillin-cupric ion interactions via standard complexation methods which involve time periods greater than this are invalid.

Weiss, Fallab & Erlenmeyer (1957), for example, measured the interaction between cupric ion and the compounds I-V in unbuffered solutions at an ionic strength of 0·1.



I



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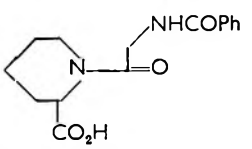

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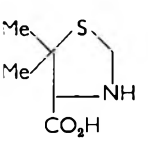
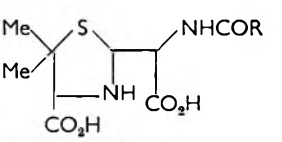


IV



V

Compounds II and III are acylated amino-acids structurally related to the penicillins I, while compound IV is structurallv related to the penicilloic acids, compound V.

The combined results for the work presented by Weiss & others (1957), and the data for this present study are given in Table 1. The value for the penicillin G-cupric ion interaction as given by the earlier work is much higher than those for the other structurally related acylated amino-acids, II and III, and higher than our values for the penicillins which, considering the ionic strength differences, are in close agreement with the values for compounds II and III. In fact, their value is not significantly different from our values for the penicilloic acids, or their values for compound IV which is more nearly related structurally to the penicilloic acids than to intact penicillin. The comparisons in Table 1 and the

TABLE 1. ASSOCIATION CONSTANTS FOR THE INTERACTION BETWEEN PENICILLINS AND RELATED COMPOUNDS WITH CUPRIC ION. DATA FOR THE PRESENT STUDY OBTAINED AT 30·00 ± 0·02° AT AN IONIC STRENGTH OF 0·01

	Compound	log K
I	Penicillin G	4·8*
II	*N*-Hippuryl-thiazolidin-4-carboxylic acid	1·8*
III	*N*-Hippuryl-pipecolinic acid	2·1*
I	Penicillin G	2·61
I	Penicillin V	2·24
IV	5,5-Dimethyl-thiazolidin-4-carboxylic acid	4·4*
V	Penicilloic V acid	4·20
V	Penicilloic G acid	4·50

* Taken from the data reported by Weiss & others (1957), at 2 ± 22° and ionic strength approximately 0·1.

very rapid rate of hydrolysis of the penicillins in the presence of cupric ion into the corresponding penicilloic acids, would suggest that the penicillin solutions used by Weiss & others had substantially degraded into the penicilloic acid during the complexation measurements.

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The determination of nonylphenol ethylene oxide detergents in wool grease*

C. A. ANDERSON, R. G. GANLY AND G. F. WOOD

The nonylphenol ethylene oxide nonionic detergent is extracted from wool grease by methanol under reflux and estimated from the infrared absorption at 1510 cm^{-1}. The method estimates between 90 and 100% of the detergent present and is independent of the type of grease. Commercial wool greases recovered from nonionic-detergent wool-scouring liquors contained between 0·5 and 2·4% detergent.

WOOL grease, the raw material for the manufacture of lanolin, is usually recovered by centrifuging wool-scouring liquors. When soap is used for scouring, the main contaminant of the grease is fatty acid, which can be readily determined (Sallee, 1958) and removed during refining. Soap has now largely been replaced by nonylphenol ethylene oxide (NPEO) nonionic detergents which contaminate the grease and adversely affect its emulsifying power. The extent of the contamination in either the crude grease or lanolin has not been previously reported.

We have examined the shortcomings of existing methods for determining the amount of NPEO detergent in the presence of wool grease and we describe a new method based on solvent extraction of the detergent and spectroscopic determination.

Experimental

MATERIALS

The wool greases used were: Sample A, lanolin, B.P. quality, supplied by Lanolin Products Pty. Ltd., Victoria. Sample B, commercial wool grease obtained by extracting Merino wool with white spirit. Sample C, freshly secreted wool grease obtained by extracting the base portion of Merino wool with diethyl ether. Sample D, the diethyl-ether extract of the tip portion of Merino wool. Sample E, obtained by refluxing grease B (25 g) with methanol (100 ml) for 1 hr and recovering the soluble fraction by decantation. All solvents and reagents were Analar grade. The detergent used in the investigation was Lissapol N450(I.C.I.) an NPEO detergent containing an average of 9·5 ethylene oxide units per molecule.

METHODS

For infrared spectroscopic analysis, wool grease samples were dissolved in chloroform (10% w/v). The spectra were measured on a Beckman IR-5 spectrophotometer from 2000–1400 cm^{-1} against a chloroform blank using 0·2 mm sodium chloride cells. The absorption due to the substituted aromatic nucleus in the detergent was calculated by subtracting

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* The term wool grease is used for the lipid material contained in the fleece. In its refined form, wool grease is designated as lanolin.

the background absorption, obtained by means of a tangent connecting the maxima flanking the analytical band, from the absorption at 1510 cm^{-1}. The concentration of detergent was then calculated from the calibration curve shown in Fig. 1.

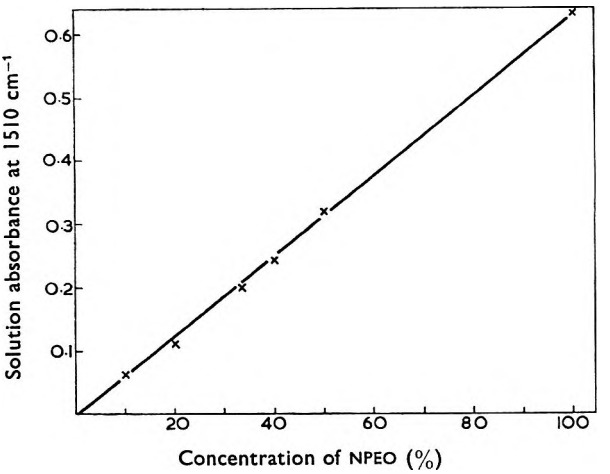

FIG. 1. Calibration curve for determining concentration of nonylphenol ethylene oxide detergent in wool grease (10% w/v solution in chloroform measured against chloroform blank). (Absorbance = $\log_{10}$1/Transmittance.)

Results and discussion

ESTIMATION OF NONIONIC DETERGENT IN WOOL GREASE BY ESTABLISHED TECHNIQUES

The quantitative determination of NPEO detergents in the presence of other compounds may be made in (i) aqueous solutions, (ii) mixed aqueous–non-aqueous solutions, and (iii) non-aqueous solutions. The methods have been reviewed by Rosen & Goldsmith (1960).

When aqueous solutions alone are used, the wool grease, which is insoluble and difficult to emulsify, is precipitated in the gravimetric methods or interferes in the volumetric and colorimetric methods. Errors are therefore introduced.

The second general technique involves reacting the detergent with ammonium cobaltothiocyanate to form a coloured complex which is extracted from the aqueous solution with chloroform or benzene, and estimated by measuring the absorption at either 620 or 318·5 mμ. The basic method was developed by Gnamm (1941) and modified by Brown & & Hayes (1955), Morgan (1962), Crabb & Persinger (1964) and Greff, Setzkorn & Leslie (1965). In these methods, the detergent is usually present in aqueous solution but preliminary tests indicated that a coloured complex is obtained if an insoluble sample (grease and detergent) and an aqueous ammonium cobaltothiocyanate solution are reacted and then extracted with chloroform or benzene. As the techniques of Crabb &

Persinger (1964) and Greff & others (1965) were the most promising, they were modified to determine NPEO detergent in wool grease.

Analysis by the techique of Crabb & Persinger (1964). In this method. the detergent is extracted from an aqueous test sample with diethyl ether, The ether solution is evaporated to dryness and ammonium cobaltothiocyanate is added to the residue. As mixtures of detergent and wool grease are not separated completely by solvent extraction, the method will be accurate only if wool grease does not interfere with the colour-forming reaction. Results obtained from various mixtures of detergent (0–5 mg) and grease B (0–500 mg) are shown in Fig. 2. Clearly the method is

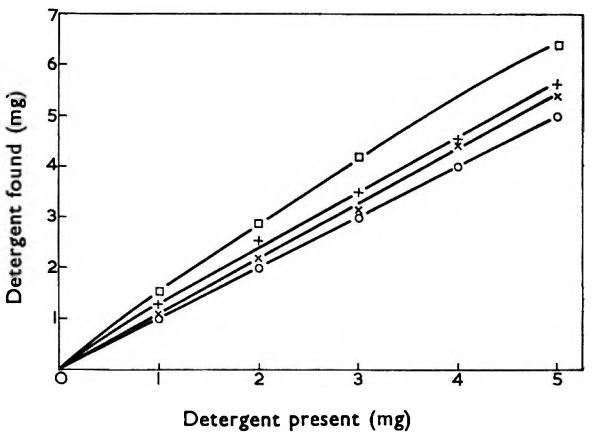

FIG. 2. The effect of wool grease B on the estimation of NPEO detergent by the method of Crabb & Persinger. ○, No added grease; ×, grease:detergent, 0·5:1; +, grease:detergent, 20:1; □, grease:detergent 100:1.

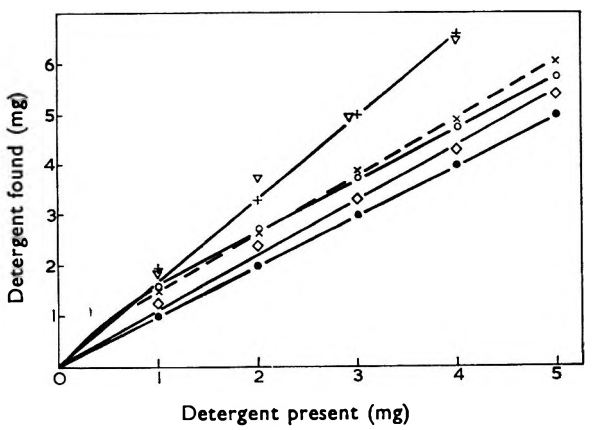

FIG. 3. The effect of various wool greases on the estimation of NPEO detergent by the method of Crabb & Persinger. ●, No added grease; ○, grease C:detergent, 100:1; +, grease D:detergent, 100:1; ▽, grease E:detergent, 100:1; ×, grease D:detergent, 0·5:1; ◇, grease E: detergent, 0·5:1.

accurate with detergent alone; however, when wool grease is present the experimental value is in error to an extent depending on the grease : detergent ratio. At high ratios (100 : 1), such as in commercial greases, the error is approx. 28%. Even when the ratio is reduced to 0·5 : 1 the error is still significant at 8%. The effect also varies with the type of grease present (Fig. 3). Thus, at a ratio of 100 : 1, the errors are 19, 66 and 62% for greases C, D, and E respectively. It is therefore impossible to apply a correction factor and the method is unsuitable for estimating small amounts of NPEO detergent in commercial wool grease.

Analysis by the technique of Greff & others (1965). In this method, ammonium cobaltothiocyanate solution is added to an aqueous solution of the detergent and the resulting coloured complex is extracted with benzene. The detergent concentration is then calculated from the extinction coefficient of the complex at 620 or 318·5 mμ. For test solution (100 ml) containing 0–25 mg the method is accurate to 10 ±3%. However, when the test solution contains wool grease also, serious errors occur (Table 1).

TABLE 1. THE EFFECT OF GREASE* ON THE ESTIMATION OF NPEO DETERGENT BY THE TECHNIQUE OF GREFF & OTHERS (1965)

Detergent present (mg)	Detergent found (mg)	
	at 620 mμ	at 318·5 mμ
24·3	77	97
12·2	34	45
4·9	13·0	19·0
2·4	6·2	9·3
0·49	0·79	1·6

*The ratio of wool grease : detergent in the test solution was 92 : 1.

Estimation of NPEO *detergent by ultraviolet spectroscopy.* Griffith's (1957) method in which the detergent-containing solid is dissolved in ethanol and the detergent content estimated from the ultraviolet absorption avoids the difficulties of the preceding methods. However, a prerequisite is that any non-detergent components present should not absorb at the selected wavelength. Measurement of the absorptions of various wool greases at the optimum wavelength for NPEO detergent estimation shows that E (1%, 1 cm) at 275 mμ for detergent and greases A, B, C, D and E are 26, 2·5, 7·0, 1·6, 11·4 and 25 respectively. Therefore even if the grease : detergent ratio could be reduced to 0·5 : 1, the error in the detergent estimation would be between 12% and 190% depending on the type of grease present.

ESTIMATION OF NONIONIC DETERGENT BY SOLVENT EXTRACTION AND INFRARED SPECTROSCOPY

A comparison of the infrared spectra of various wool greases and an NPEO detergent (Fig. 4) indicates that the absorption at 1510 cm^{-1}, due to the substituted aromatic nucleus, could be used to estimate the detergent if a suitable preliminary concentration could be effected. As methanol

under reflux extracts the relatively small quantity of polar components from wool grease (Anderson & Wood, 1965), it seemed likely that the detergent might also be extracted. Various parameters of extraction were therefore examined to find the optimum conditions. The findings showed that a maximum recovery of 95% is obtained at a solvent : grease ratio of 4:1 after refluxing for 1 hr. For estimation of detergent, the methanol-soluble fraction was evaporated on a rotary vacuum evaporator and dried *in vacuo* for 30 min. The residue was dissolved in chloroform and the absorption at 1510 cm^{-1} determined.

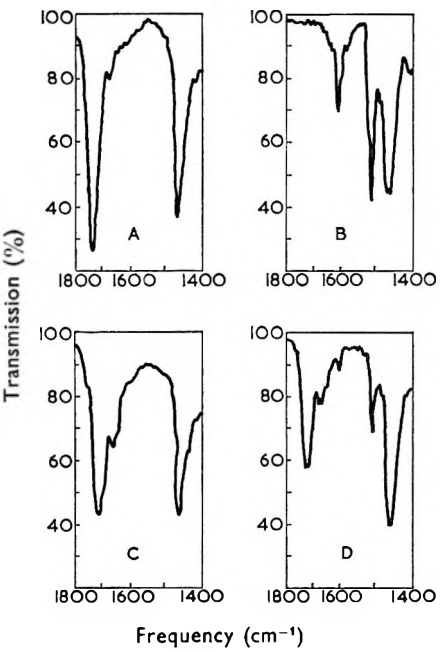

FIG. 4. Infrared spectra of Lissapol N450 and various wool greases: A, commercial centrifuged grease from soap scouring containing no detergent; B, Lissapol N450; C, methanol-soluble fraction of grease used for spectrum A; D, methanol-soluble fraction of commercial centrifuged grease from NPEO scouring. Solutions (5% w/v was used for illustrative purposes) in chloroform were measured against a chloroform blank.

RECOMMENDED PROCEDURE

To anhydrous grease (10·00 g) add methanol (40 ml) and reflux vigorously for 60 min. Cool the mixture to about 20° and then decant the supernatant liquor and flocculent material* into a tared flask. Wash the insoluble grease with cold methanol (2 × 10 ml) and add the washings also to the tared flask. Evaporate the combined methanol solution to dryness. Dissolve the residue in chloroform (10·00% w/v) and determine the absorption at 1510 cm^{-1}, using a 0·2 mm sodium chloride cell. From

* If this is not done about 3% of the detergent will be lost.

the calibration curve (Fig. 1) read off the concentration of detergent in the methanol-soluble (MS) fraction. Detergent concentration in grease =

$$\frac{\text{Detergent in MS} \times \% \text{ MS}}{100}$$

To establish the accuracy of the method, fourteen mixtures of Lissapol N450 with samples A and B were analysed. The results indicated that the method has an accuracy of 95 $\pm$5%. Some typical figures are shown in Table 2.

TABLE 2. DETERMINATION OF DETERGENT CONTENT OF VARIOUS GREASES BY INFRARED SPECTROSCOPY

Detergent conc. (found) (%)	0·55	0·80	1·20	1·95	3·31	4·90
Detergent conc. (actual) (%)	0·55	0·85	1·30	2·03	3·51	5·20

Checks with several NPEO detergents from different manufacturers showed that those normally recommended for wool scouring have 8·5–10 ethylene oxide units in the chain and give virtually the same absorption at 1510 cm^{-1}.

DETERGENT IN COMMERCIAL GREASES

Using the above procedure, the NPEO detergent contents of commercially recovered greases were determined. Twenty samples from 3 different wool-scouring mills contained amounts of detergent varying from 0·55 to 2·4%. It is not known at present whether subsequent refining will remove detergent, but preliminary experiments indicate that water-washing is ineffective.

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Apparatus for accelerated temperature cycling

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An automatic control unit, activating electrically operated solenoid valves, is used to regulate the flow of "hot" and "cold" water from two thermostated baths to the jacket of a reaction vessel so that the temperature of a liquid sample within the vessel follows a highly reproducible programmed cycle. A comparison is made of the temperature hysteresis between 23–33° during a 16 min cycle, when the flow rates to the vessel are changed and when the valves are operated manually. The adjustment of temperature hysteresis to effect the accelerated storage testing of liquid preparations is described.

IN recent years the method of choice for the accelerated storage testing of pharmaceutical preparations has been the exposure of several different samples of the same preparation to one of a number of elevated temperatures (Garrett, 1962). This technique is applicable to chemical degradation only and produced mathematically satisfactory results on the basis of the Arrhenius equation. Nevertheless, protracted shelf storage tests under ambient conditions were still necessary to ensure that the experimental results correlated with shelf storage conditions.

The physical degradation of a product is more likely to occur under temperature oscillation than at a static elevated temperature. Thus, high frequency, reproducible temperature cycling is desirable for producing the accelerated physical degradation.

Several designs of temperature controller were investigated, but none was entirely suitable in producing the rapid temperature cycling of liquid preparations (Brandt & Brown, 1955; Barnes, 1956; Beament & Machin, 1959; Boer, 1963; Scott, Min, Campbell & Anderson, 1964; Van Outryve, 1965; Cole, Dickinson, Guczi, Hill & Tyrrell, 1965; Borgars, 1966).

We now report a design which, because of good heat transfer properties, permits a relatively high frequency of temperature cycling and the examination of one sample of the preparation over very short periods. Thus, it is possible to produce physical changes in the preparation by the variation of the time factor alone without recourse to large numbers of samples, elevated temperatures (which may alter the mechanism of degradation) or bulky apparatus.

APPARATUS

The basic apparatus is a stainless steel, jacketed reaction vessel connected to two thermostated baths supplying "hot" and "cold" water (Carless & Foster, 1966).

Water circuitry. The suspension to be studied is placed in the vessel and the temperature cycling is induced by the passage through the vessel jacket of "hot" water and "cold" water alternately, each supplied from a thermostatic bath fitted with an external pump (Circotherm II*). Suitable

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* Shandon Co., Ltd. London.

adjustment of the temperature and flow rates from each bath permits the temperature cycle to conform to the preselected programme, e.g. 23–33–23° during times 0–8–16 min. Bypasses are provided, so that, when the "hot" water is flowing through the vessel the "cold" water is being pumped through the bypass, and vice versa. By this means, it is possible to maintain a constant pumping rate for each bath through the temperature cycle. Fig. 1 illustrates the water circuitry to effect these conditions and

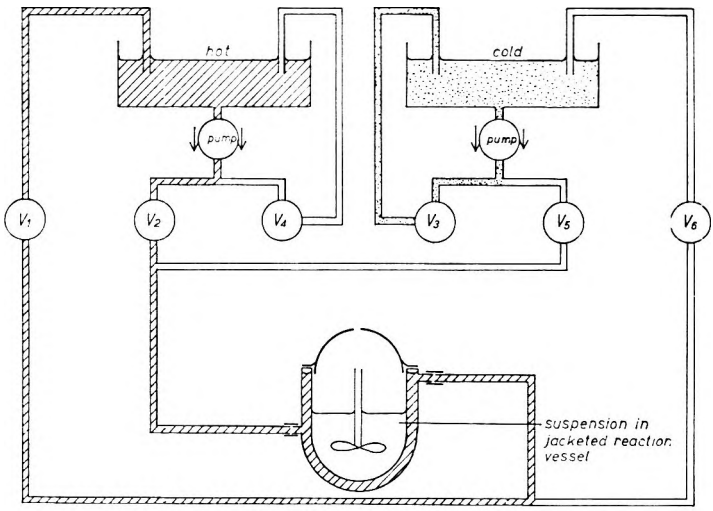

FIG. 1. Flow of hot water through reaction vessel.

shows the suspension being heated. V_1 to V_6 inclusive are solenoid-operated water valves, which may be either in the open or closed position. Valves V_1, V_2 and V_3 are open when the suspension is being heated, whereas V_4, V_5 and V_6 are open when the suspension is being cooled.

V_3 and V_4 are open when both water supplies are allowed to bypass the reaction vessel. This condition permits the baths to acquire their working temperatures before a cycling run is commenced.

The Cyclothermostat. In the initial work (Carless & Foster, 1966) using the water circuitry described, the valves V_1 to V_6 were operated manually every 8 min. This did not permit easy operation, particularly over protracted periods, so the valve operation was automated by the introduction of the "Cyclothermostat", the circuitry of which is shown in Fig. 2. S_1 to S_6 inclusive represent a six-pole, three-way switch and the three positions used are, as indicated on the figure, OFF/BYPASS/RUN.

In the position OFF the power is essentially disconnected. For BYPASS power is supplied to valves V_3 and V_4 (neons P_8 and P_7); also by operation of the relay A/2 (contacts A_1 and A_2), power is supplied to the thermostatically controlled heaters and pumps. In the BYPASS position the thermostatic baths are allowed to attain their working temperatures.

On RUN, the motor, M, starts to turn and the neon lamp, P_2, indicates that power is being supplied to the motor. The microswitch, S_8, is positioned so that it operates valves V_4, V_5 and V_6 (neons P_7, P_5 and P_6). The counter operates and a visual record of the number of the cycle is registered. S_7 is an "autoreset" microswitch, which actuates the counter during a cycling run and which is also used to switch off the motor at the

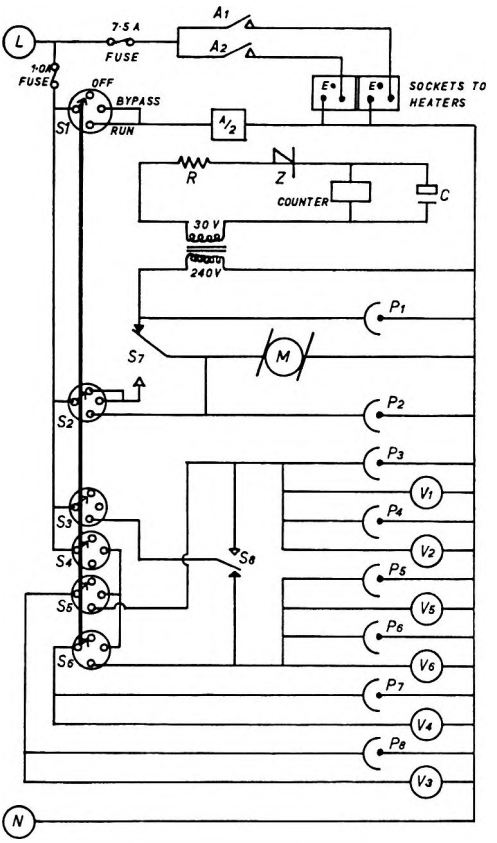

FIG. 2. Circuit diagram of the cyclothermostat. Components: P_1 to P_8, neon indicator lamps 712D (Bulgin). V_1 to V_6, Alcon ACO2 solenoid-operated valves (Alexander Controls) (numbered as in Fig. 1). S_1 to S_6, six-pole, three-way switch S438 (Bulgin). S_7, microswitch S511 (Bulgin). S_8, microswitch S511/RSS (Bulgin). M, Sangamo Weston motor, 12 r.p.h. (M. R. Supplies) (Gearing, 20:64). Counter, P.O. Counter, 24 v, S375 (H. Franks). Transformer, 240 v/30 v a.c. C, 25 mfd, 50 v wkg (Radiospares). R, 20 ohm Hystab (Radiospares). Z, REC 50A (Radiospares). A/2, Relay Type 12, 5A contacts (Radiospares).

end of a run. Since the same motor, is used for microswitch, S_8, the "autoreset" switch always switches off the valve control at the same point in the cycle, when a run is completed. Thus, at the beginning of a run, S_8 is always in the position shown and valves V_4, V_5 and V_6 are open.

The "autoreset" switch is so positioned that, within 15 sec of switching to "RUN", S_8 changes, V_1 V_2 and V_3 (neons P_3, P_4 and P_8) are opened and a

heating phase starts. This position corresponds to a time of 15¾ min in Fig. 3.

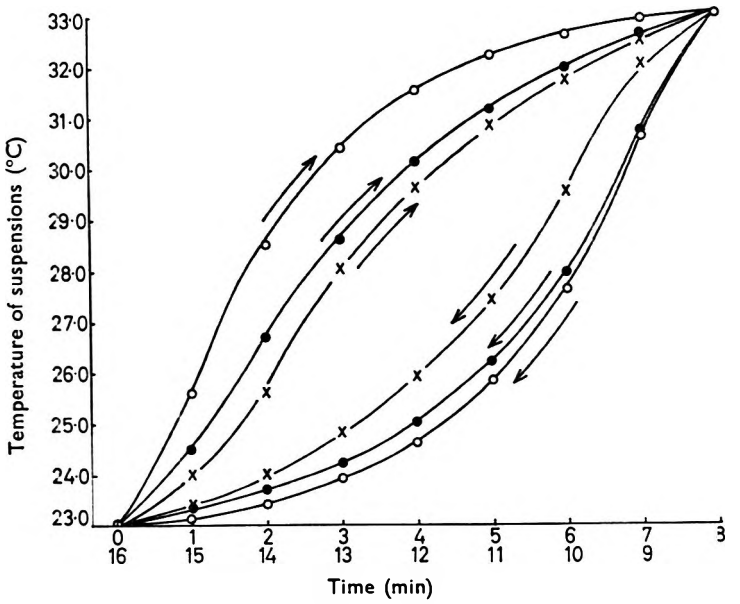

FIG. 3. Suspension temperature during cycling. ○ = 740 ml/min, ● = manual, X = 320 ml/min.

Neon lamp, P_1, is connected through S_7 so that it lights as the microswitch is tripped, giving a visual signal 15 sec before a heating phase is about to commence. During this time the temperature of the solution or suspension in the reaction vessel is approaching 23° and a sample may be removed with a pipette.

The supply sockets for the thermostatically-controlled heaters are included in the wiring diagram of the Cyclothermostat so that they are switched on at the same time as the valves and counter, thus the Circotherm pumps do not pump against closed valves.

SOME RESULTS

The effect of passing "hot" water and "cold" water through the vessel jacket is to produce a temperature hysteresis in *the bulk of the suspension.* Fig. 3 illustrates the temperature hysteresis observed and each curve represents results from duplicate experiments. The variation between duplicate results was always less than 0·05°.

All three sets of curves conform to the preselected programme of 23–33–23° in a 16 min cycle, divided into an 8 min heating phase and an 8 min cooling phase. The curves annotated "manual" were obtained using the manual version of the instrument (Carless & Foster, 1966).

The other two sets of curves were obtained from Cyclothermostat controlled experiments with vehicle flow rates of 320 ml and 740 ml/min.

The relative and actual flow rates of the "hot" and "cold" vehicle were adjusted by introducing suitable lengths of capillary tubing into each side of the water circuitry. By manipulation, it was possible to obtain a temperature hysteresis identical with manual operation results (not illustrated).

Other factors which may influence the hysteresis are (a) the volume of sample, (b) relative and actual times of each phase of the cycle, (c) sample stirring rate and the degree of agitation within the reaction vessel jacket, (d) ambient laboratory temperature, insulation of the reaction vessel and the temperature range chosen.

Most of the control of the hysteresis is readily achieved by altering the temperature settings in the thermostatically controlled baths, altering vehicle flow rates and interchanging the motor and cam mechanism.

Improvement in thermostat control may be achieved by the method of Finch (1963) and an automatic-thermostat-warming-up-device may be employed to permit the baths to attain their working temperatures more quickly (Jervis, 1955).

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Hypotensive action of tyramine in cats

SIR,—During experiments on the interaction between cheese and monoamine-oxidase inhibitors in cats (Maj & Langwiński, 1966) we noticed that repeated administration of tyramine decreased the blood pressure. We now report on investigation of this depressor action.

The experiments were made on 38 cats anaesthetised with chloralose (80 mg/kg, i.p.), most of them pretreated with nialamide (20 mg/kg, i.p.). Blood pressures were recorded from the carotid artery by a mercury manometer and all the substances were injected into a femoral or jugular vein.

Repeated doses of tyramine were given until complete tachyphylaxis to its pressor effect was obtained, the total dose being 4–8 mg/kg. After this, the subsequent injection produced only a depressor response. The minimal hypotensive dose of tyramine was 1–3 mg/kg. Higher doses (10–50 mg/kg) caused a 30–70 mm Hg fall in blood pressure. The onset of the depressor response was delayed for 15–30 sec, and the minimal level was seen 1–2 min after injection and then the blood pressure increased gradually to reach the initial value within 5–15 min. In some cats, after the large doses, the blood pressure did not return at all. The hypotensive effect was observed also in cats not treated with nialamide, but under these conditions the total dose of tyramine needed to induce the blood pressure decrease was 3–5 times greater. After reserpine (3 mg/kg, i.p.) injected one day before, the depressor response to tyramine appeared more rapidly than in untreated animals.

The hypotensive action of tyramine was not influenced by bilateral cervical vagotomy, pretreatment with atropine sulphate (0·5 mg/kg) blockade of the α-adrenergic receptors with dihydroergotamine (0·3 mg/kg) or of the β-adrenergic receptors with dichloroisoprenaline (5–10 mg/kg). It was antagonised only by the antihistamine drugs, antazoline hydrochloride (10–30 mg/kg) and cyclizine hydrochloride (2 mg/kg).

The depressor response to tyramine has also been observed after repeated doses in rats anaesthetised with urethane (1 g/kg), especially in those previously treated with nialamide. The doses of 2–8 mg/kg produced a 20–70 mm Hg blood pressure fall. Atropine had no influence on this action, but antazoline abolished it.

Tyramine (5×10^{-4}) contracted the isolated guinea-pig ileum, an effect partially antagonised by atropine (10^{-6}). Antazoline (10^{-6}—10^{-5}) protected the atropinised ileum completely against the contractive action of tyramine.

The results of our experiments on the depressor response to tyramine, especially the delayed onset and the antagonistic effect of antihistamine drugs seem to indicate that this action of tyramine is mediated through the release of histamine. The fact that the response was more pronounced in cats pretreated with monoamine oxidase inhibitor may be a consequence of a simultaneous diamine oxidase blockade, well known from the literature. In fact we have observed a stronger hypotensive action of exogenous histamine in these cats.

The hypotensive action of tyramine in some species has been reported by several authors. Dresse & Cession-Fossion (1961) observed it in rats pretreated with guanethidine. Maxwell & others (1959) observed it in reserpinised dogs, but they did not analyse it. Vanderipe & Kahn (1964) reported a number of facts indicating that the depressor response to tyramine in dogs depends on the histamine liberation. According to Chandra, Dhawan & Gupta (1965) this response in dogs may be induced by the release of acetylcholine. Only Paton (1957) suggested the histamine releasing properties of tyramine in cats from the skin preparation.

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The effect of ethanol on the activity of central catecholamine neurones in rat brain

SIR,—There are no apparent changes in the dopamine, noradrenaline or 5-hydroxytryptamine levels in rabbit and rat brain after administration of ethanol (Häggendal & Lindqvist, 1961; Efron & Gessa, 1963). But although the amine levels are unaffected, this does not exclude the possibility that the activity of the central monoamine neurones may be influenced by ethanol. As a result of the development of inhibitors of the rate-limiting step in catecholamine synthesis it has now become possible to examine the activity of the central catecholamine neurones directly at cellular level by the histochemical fluorescence technique (Hillarp, Fuxe & Dahlström, 1966). These experiments showed that the release and synthesis of amine is dependent on neuronal activity (Fuxe & Gunne, 1964; Dahlström, Fuxe, Kernell & Sedvall, 1965; Andén, Corrodi, Dahlström, Fuxe & Hökfelt, 1966; Corrodi & Malmfors, 1966). H 44/68 (DL-α-methyl-tyrosine-methylester) used in this and previous studies (Corrodi, Fuxe & Hökfelt, 1966a) inhibits the biosynthesis of noradrenaline and dopamine without affecting the uptake-storage mechanism of the amine granules (Andén & others, 1966; Corrodi, Fuxe & Hökfelt 1996b; Corrodi & Hanson, 1966).

The present communication affords evidence that changes do occur in the central catecholamine neurones during treatment with ethanol as revealed by both histochemical and biochemical techniques.

Male, Sprague-Dawley rats (150–250 g) were treated with ethanol (2 g/kg as a 5% solution i.p. and H 44/68 (250 mg/kg i.p.). Some animals were given one injection of ethanol and this was followed by H 44/68 15 min later. The rats were then killed 2, 4 or 6 hr later. Other animals were given two doses of ethanol, H 44/68 was administered to these 15 min after the first dose and 4 hr before death; the second dose of ethanol was given 1½ hr before death by which time the animals were asleep without a righting reflex. The whole brains were dissected and analysed separately for dopamine and noradrenaline (Bertler, Carlsson & Rosengren, 1958; Carlsson & Waldeck, 1958; Carlsson & Lindqvist, 1962). Control rats were given either ethanol or H 44/68. The rectal temperature in all animals was found to be normal. After the ethanol the animals showed no signs of peritoneal pain nor did the peritoneal cavity show inflammation.

In the histochemical study the effect of two different doses of ethanol (1 and 2 g/kg) was investigated. Ethanol was administered intraperitoneally once or twice as described above. The animals were killed 4 hr after the i.p. injection of H 44/68 (250 mg/kg) which was given 15 min after the ethanol. Other rats were given ethanol (2 g/kg) by mouth and after H 44/68 treatment as described they

were killed. Control rats were treated with either ethanol or H 44/68. The animals were decapitated under light chloroform anaesthesia; the medulla oblongata, the pons, the mesencephalon, the diencephalon and large parts of the telencephalon were dissected, freeze-dried, treated with formaldehyde gas for 1 hr, embedded in paraffin, sectioned and mounted (Dahlström & Fuxe, 1964; Hamberger, Malmfors & Sachs, 1965).

After treatment with ethanol alone no significant changes were observed in the catecholamine levels of the brain. But, in the animals also treated with H 44/68, there was a greater decrease of noradrenaline but not dopamine in the brain compared with the rats receiving only H 44/68 (Table 1). After two doses of

TABLE 1. NORADRENALINE AND DOPAMINE CONCENTRATIONS IN RAT BRAIN 2, 4 AND 6 HR AFTER ETHANOL (2 G/KG, I.P.) AND H 44/68 (250 MG/KG, I.P.) 15 MIN LATER.
(percent of normal values ± s.e.m.)

Treatment	No. of experiments	Noradrenaline %	Dopamine %
Untreated	30	100·0 ± 2·0	100·0 ± 2·5
2 hr			
Ethanol	4	94·3 ± 3·2	95·0 ± 5·1
H 44/68	4	75·4 ± 1·3	41·9 ± 2·9
Ethanol + H 44/68	4	66·7 ± 2·4	47·2 ± 3·8
		• (P < 0·01)	
4 hr			
Ethanol	6	92·1 ± 2·2	88·7 ± 6·2
H 44/68	6	58·7 ± 1·5	26·9 ± 3·0
Ethanol + H 44/68	6	44·7 ± 2·4	27·8 ± 2·2
		• (P ~ 0·001)	
6 hr			
Ethanol	4	93·2 ± 3·1	90·0 ± 3·1
H 44/68	4	41·5 ± 1·3	17·4 ± 2·8
Ethanol + H 44/68	4	38·9 ± 1·3	22·2 ± 2·5

TABLE 2. NORADRENALINE AND DOPAMINE CONCENTRATIONS IN RAT BRAIN 4½ AND 1½ HR AFTER ETHANOL (2 G/KG, I.P.) AND 4 HR AFTER H 44/68 (250 MG/KG I.P.).
(Percent of normal values ± s.e.m.)

Treatment	No. of experiments	Noradrenaline %	Dopamine %
Untreated	30	100·0 ± 2·0	100·0 ± 2·5
Ethanol	4	87·4 ± 1·9	98·8 ± 5·0
H 44/68	4	54·0 ± 2·8	29·2 ± 2·7
Ethanol + H 44/68	4	33·6 ± 3·1	22·6 ± 1·2
		• (P ~ 0·0005)	

ethanol this decrease of noradrenaline was greater (Table 2). The difference between the test and control animals was most marked 4 hr after H 44/68 injection, somewhat less after 2 hr and not significant after 6 hr. No definite effects could be observed histologically in the dopamine and noradrenaline levels of central monoamine neurones in the parts of the brain 4 hr after ethanol. In the animals given the higher dose of ethanol intraperitoneally twice with H 44/68, the reduction of amine fluorescence in the specific noradrenaline—but not in the dopamine—nerve terminals of the brain was more marked than that observed after treatment with H 44/68 alone. After a single dose of 2 g/kg an accelerated depletion of noradrenaline was observed only in *some* rats, while others showed no definite changes in the rate of fluorescence disappearance compared to controls. Most of the noradrenaline nerve terminal systems of the brain seemed to be affected, e.g. those innervating the nucleus supraopticus,

nucleus paraventricularis, the preoptic area, the nucleus tractus solitarii and nucleus motorius dorsalis n. vagi. The amine levels of the nerve cell-bodies in the various catecholamine cell-groups showed about the same decreases after H 44/68 treatment whether ethanol had been given or not.

The present findings demonstrate that the central noradrenaline neurones are specifically activated, directly or indirectly, 2 to 4 hr but not 6 hr after acutely administered ethanol. This increase in activity would thus result in an increased release and synthesis of noradrenaline which could be revealed after inhibition of synthesis. These findings may explain the inhibitory effects of ethanol on antidiuretic hormone secretion (Hirvonen, Karlsson & Virtanen, 1966) and on oxytocin secretion (Fuchs, 1966), since the noradrenaline nerve terminals surrounding these nuclei probably are inhibitory in function (Fuxe & Hökfelt, 1966). Whether this central effect of ethanol and its known effect on animal and human behaviour to related is not yet known.

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Potentiation of noradrenaline toxicity by drugs with antihistamine activity

SIR,—The finding that antihistamine drugs potentiate the cardiovascular action of noradrenaline (Sherrod, Loew & Schloemer, 1947; Innes, 1958) has recently been interpreted in terms of an inhibition of noradrenaline uptake by peripheral tissues (Isaac & Goth, 1965).

While examining the interaction of drugs blocking catecholamine uptake with the changes of body temperature induced by noradrenaline and reserpine it was observed that in relatively low doses these drugs also increased the toxicity of noradrenaline.

Female Sprague-Dawley rats (150 ± 5 g) in groups of at least 5 for each dose, were kept at 22° and a relative humidity of 60%. They were treated intraperitoneally with the drugs 1 hr before receiving different doses of noradrenaline. Where death occurred was it almost immediate, but the LD 50 (Litchfield & Wilcoxon, 1949) was calculated from dose-mortality data obtained 24 hr after the treatment.

The drugs used were: (+)-chlorpheniramine hydrochloride, tripelennamine hydrochloride, desipramine, pyrilamine hydrochloride, noradrenaline bitartrate.

The results in Table 1, show that (+)-chlorpheniramine, tripelennamine and

TABLE 1. THE EFFECT OF DRUGS WITH ANTIHISTAMINE ACTIVITY ON THE TOXICITY OF NORADRENALINE GIVEN INTRAPERITONEALLY TO RATS 1 HR AFTER THE DRUGS

No. of rats	Drug pretreatment 7·5 mg/kg i.p.	LD 50 Noradrenaline (mg/kg i.p.)	95% confidence limits
18	Control	8·0	7·41–8·64
24	(+)-Chlorpheniramine	3·2	2·5 –4·1
51	Desipramine	4·1	3·46–4·98
33	Tripelennamine	5·3	4·45–6·3
30	Pyrilamine	8·2	7·19–9·35

desipramine, but not pyrilamine, significantly increased the toxicity of noradrenaline.

Desipramine shows a slight antihistamine property but it is a powerful inhibitor of the catecholamine uptake process (Glowinski & Axelrod, 1964; Iversen, 1965); on the contrary, pyrilamine exerts greater antihistamine activity than the other compounds, but it was inactive on the uptake of tritiated noradrenaline by the isolated rat heart (Isaac & Goth, 1965).

Thus antihistamine potency appears not to be related to the modified responses to noradrenaline. But some antihistamine drugs, blocking the uptake mechanisms, are able to intensify the effect and to enhance the toxicity of exogenous noradrenaline. This fact might be responsible for undesirable side-effects seen on occasions in clinical treatment, where antihistamines are given with sympathomimetic agents.

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The absorption of carrageenans

SIR,—An understanding of the manner of gastrointestinal absorption of carrageenans would be useful in two ways. First, it would add to knowledge of the absorption of sulphated polysaccharides, including heparin, which is present on both sides of the gastrointestinal wall, but appears not to be absorbed in active form or quantity. Second, it would help to explain the actions of carrageenan in reducing histamine-induced parietal cell hyperplasia (Anderson & Soman, 1965a), in reducing secretion (Anderson, Marcus & Watt, 1962), and in preventing histamine-induced gastric ulceration in the pylorus-ligated guinea-pig when administered intraduodenally (Anderson & Soman, 1963). The last-named action indicated for the first time that the gastric effects of carrageenans (principally, anti-ulcer and anti-secretory) could be accomplished either after absorption or by one of several possible humoral effects (Giertz, Hahn, Schmutzler & Seseke, 1964; Anderson & Soman, 1966).

Carrageenans Degraded κ-carrageenan from *Eucheuma spinosum* (C 16), degraded λ-carrageenan from *Gigartina pistillata* (GP-λ-D2), and undegraded λ-carrageenans from *Chondrus crispus* and *G. Pistillata* (CY-λ and GP-λ respectively) were used. Weight-average molecular weights (light-scattering) for the degraded carrageenans are 20,000–30,000; for the undegraded carrageenans they are 800,000–1,000,000.

Administration of carrageenans. Single intravenous injections of saline solutions of C 16 were given to male guinea-pigs, average weight 400 g, of P strain (Anderson & Soman, 1966) food, but not water, was withheld for 18 hr and at the end of this period urine was manually expelled from the bladder (or removed immediately after killing) and examined for sulphated polysaccharide. Qualitative examination was by staining with toluidine blue on filter paper; quantitative examination was by MacIntosh's (1941) method, suitably modified.

Oral administration was by inclusion in the drinking water, the initial and residual volumes over 18 hr being recorded. Food was withheld during the period. Oral consumption was also measured during experiments designed to demonstrate the anti-duodenal ulcer activity of carrageenan. Duodenal ulceration was produced by histamine acid phosphate (10 mg/kg in a wax-oil base, intramuscularly) (Anderson & Soman, 1965b).

Intraduodenal administration in 2 ml of saline was effected during anaesthesia (pentobarbitone sodium, 30 mg/kg, intraperitoneally) in the pylorus-ligated guinea-pig in an experiment designed (Anderson & Soman, 1963), to demonstrate the anti-gastric ulcer effect of carrageenan.

The results of intravenous injection of C16 are in Table 1. Increase in dose of

TABLE 1. URINARY CONTENT OF DEGRADED CARRAGEENAN AFTER A SINGLE INTRAVENOUS INJECTION TO GUINEA-PIGS

Group	Number of animals	Dose of degraded carrageenan mg/kg i.v.	Degraded carrageenan in urine mg/ml
1	4	2·5	0 (but present qualitatively)
2	3	4	0·03
3	4	5	0·05
4	3	5	0·03
5	4	7·5	0·13
6	5	7·5	0·11
7	3	10	0·07
8	3	12·5	0·06
9	3	15	0·33

C16 corresponds roughly to increase in urinary content of the polysaccharide. Assuming that it is degraded carrageenan which appears in the urine, the results show that between 2·5 and 5 mg/kg C16 given intravenously to the guinea-pig results in the apppearance of urinary degraded carrageenan, indicating that there is no total renal barrier to its excretion. A method depending on metachromasia indicates that the polyanionic nature of the carrageenan is unchanged during its passage through the animal body.

C16 was also administered orally to guinea-pigs in drinking water (1%), over a period of 18 hr. When 298 mg of degraded carrageenan was consumed, the urine showed the qualitative presence of C16 only; using a 5% solution (1850 mg consumed) the urine contained about 0·3 mg/ml at the end of the experiment. Intraduodenal administration of 400 mg C16 in an anti-gastric ulcer experiment in the pylorus-ligated guinea-pig gave detectable, but not measurable, urinary metachromasia, indicating intestinal absorption. Gastric ulceration was reduced by 60%. GP-λ-D2 gave similar results. The experiment with GP-λ-D2 was conducted in association with an anti-duodenal ulcer test of this substance and the consumption of 675 mg of GP-λ-D2 was accompanied by 73% reduction in duodenal ulceration and demonstrable, but not measurable, urinary metachromasia. In an anti-gastric ulcer experiment the intraduodenal administration of 150 mg of GP-λ-D2 gave no demonstrable urinary metachromasia, but resulted in 47% reduction in gastric ulceration. Absorption of GP-λ-D2, if indeed it occurred, therefore appeared to be less than about 2·5 mg/kg (equivalent to about 1 mg total per animal) on the basis of the results in the Table. The minimum dose of GP-λ-D2 showing anti-gastric ulcer activity is 50 mg (Anderson & Soman, to be published); of this, it appears that about 1 mg or less can be absorbed. This suggests that the effect of carrageenan in anti-ulcer experiments is to stimulate some protective mechanism. When 400 mg of C16 was administered intraduodenally in addition to an intravenous injection (groups 4 and 6) the measurable urinary carrageenan did not increase, supporting the conclusion that intraduodenal carrageenan is absorbed in very small amounts.

With the degraded carrageenans it therefore appears that the anti-ulcer effect can be achieved with amounts of carrageenan less than that necessary to give detectable urinary excretion.

Turning to undegraded carrageenan (GP-λ), we have never detected it in urine, although the maximum total dose consumed was 158 mg. GP-λ does have an anti-ulcer effect (Anderson & Soman, to be published), but its high viscosity in solution precludes larger oral or intraduodenal dosage, which the experiments with degraded carrageenan show are necessary to obtain urinary excretion. Thus with the undegraded carrageenans also, doses giving anti-ulcer effect are lower than those which would be required to show urinary excretion. The absorption of undegraded carrageenan, although generally held to be unlikely, is still an open question on the basis of its anti-gastric ulcer effect after intraduodenal administration in the pylorus-ligated guinea-pig (Anderson & Soman, to be published). There are two further points about undegraded carrageenan: first, its molecular size (assuming absence of excessive polydispersity) may well prohibit gastrointestinal absorption; second, intravenous injections of higher doses of undegraded carrageenans in this type of experiment are prevented by extreme toxicity both in rabbits (Anderson & Duncan, 1965) and in guinea-pigs at 1 mg/kg, the animals dying, probably from pulmonary embolism, within half an hour.

That gastrointestinal absorption of degraded carrageenan is possible, helps to explain the more efficient protection (Anderson & Soman, 1965b) against experimental histamine duodenal ulceration the carrageenan gives when administered in drinking water before the histamine rather than after it.

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Effect of amphetamine and reserpine on the pressor response to tyramine in the rabbit and cat

SIR,—The potentiation of the pressor response to tyramine after amphetamine has been shown in the dog (Eble & Rudzik, 1965) and in the rat (Eble & Rudzik, 1966a). However combinations of amphetamine and reserpine antagonise the pressor response to tyramine in the dog (Eble & Rudzik, 1966b). In these studies of the interactions of amphetamine and tyramine in other species it was found that in the rabbit, tyramine was a relatively weak pressor agent and that its effect was not potentiated by amphetamine, but was blocked by combinations of amphetamine and reserpine. The pressor responses of the cat to tyramine and the interaction with amphetamine and reserpine were similar to those found in the dog but not like those found in the rabbit.

Rabbits (3-5 kg) and cats (1·8–3·6 kg) of either sex were anaesthetised with sodium pentobarbitone (32 mg/kg, i.v.), with supplements of 3·2 mg/kg as required. Blood pressures were recorded from the carotid artery and drug injections made through a polyethylene tube passed 3 to 5 cm into a femoral vein.

(+)-Amphetamine (250 or 500 μg/kg, i.v.) failed to potentiate the pressor response to tyramine in rabbits (Table 1). In two additional experiments larger

TABLE 1. EFFECT OF AMPHETAMINE AND RESERPINE ON THE PRESSOR RESPONSE TO TYRAMINE IN THE RABBIT

Treatment	No. of Determinations	Mean b.p. response to tyramine (250–500 μg/kg, i.v.)	P value
Control	5	31 ± 5·9	
After amphetamine (250 μg/kg, i.v.)		36 ± 4·9	> 0·1
After amphetamine (250 μg/kg, i.v.) + reserpine (1 mg/kg, i.v.)		51 ± 4·5	< 0·02
Control	7	21 ± 3·0	
After amphetamine (500 μg/kg, i.v.)		28 ± 4·2	> 0·1
After amphetamine (500 μg/kg, i.v.) + reserpine (1 mg/kg, i.v.)		45 ± 4·6	< 0·01
Control	5	41 ± 5·4	
After reserpine (1 mg/kg, i.v.)		56 ± 10·1	> 0·05
After reserpine (1 mg/kg, i.v.) + amphetamine (250 μg/kg, i.v.)		28 ± 3·4	< 0·05
Control	5	30 ± 5·0	
After reserpine (2 mg/kg, i.v.)		62 ± 7·6	< 0·02
After reserpine (2 mg/kg, i.v.) + amphetamine (250 μg/kg, i.v.)		30 ± 5·0	< 0·001

doses of amphetamine (1 mg/kg, i.v.) also failed to potentiate the pressor response to tyramine. The response after reserpine in the amphetamine-pretreated rabbits, was further potentiated. This differs from our earlier findings in the dog (Eble & Rudzik, 1966b) in which the response to tyramine was blocked after combinations of amphetamine and reserpine.

The pressor response to tyramine was potentiated by the acute administration of reserpine (2 mg/kg, i.v.) in the rabbit. But if the reserpine was given before a dose of amphetamine there was a diminution of the pressor response to tyramine. Hence in the rabbit, the sequence of amphetamine-reserpine appears to determine the influence of the combination on the pressor response to tyramine. We have previously found (Eble & Rudzik, 1966b) that both sequences effectively antagonise the pressor response to tyramine in the dog.

In the cat, as in the dog, rabbit and rat, the acute administration of amphetamine or reserpine potentiated the pressor response to tyramine (Table 2). The

TABLE 2. EFFECT OF (+)-AMPHETAMINE AND RESERPINE ON THE PRESSOR RESPONSE TO TYRAMINE IN THE CAT

Treatment	No. of Determinations	Mean b.p. response to tyramine in mm Hg	P value
Control	8	38 ± 4·4*	
After amphetamine (250 μg/kg, i.v.)		54 ± 5·9	< 0·002
After amphetamine (250 μg/kg, i.v.) + reserpine (1 mg/kg, i.v.)		19 ± 4·1	< 0·001
Control	5	46 ± 6·2*	
After reserpine (1 mg/kg, i.v.)		70 ± 9·2	< 0·01
After reserpine (1 mg/kg, i.v.) + amphetamine (250 μg/kg, i.v.)		28 ± 5·2	< 0·02

* The control dose of tyramine was 50–100 μg/kg, i.v.

combination of amphetamine and reserpine in both sequences, antagonised the pressor response to tyramine.

It seems that both amphetamine and reserpine can potentiate the pressor response to tyramine and that combinations of the two agents block this pressor response. The sequence of administration of amphetamine and reserpine appears to be important for this blockade in the rabbit but not in the dog, cat and rat. The failure of amphetamine to potentiate the response to tyramine in the rabbit may be related to the relative insensitivity of the rabbit to tyramine.

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Effect of pentobarbitone administration on corticosteroid-induced synthesis of hepatic gluconeogenic enzymes

SIR,—Administration of glucocorticoids in rats caused preferential increases in liver gluconeogenic enzymes without affecting other enzymes involved in carbohydrate metabolism (Weber & Singhal, 1964a; 1964b). That the steroid-induced increases observed for hepatic glucose 6-phosphatase and fructose 1,6-diphosphatase were due to new enzyme synthesis was revealed in recent investigations in which actinomycin, puromycin and ethionine abolished these increases (Weber & Singhal, 1964b; Weber, Singhal & Srivastava, 1965a). More recently, evidence has been obtained that glucocorticoid hormones function as inducers and insulin as a suppressor of biosynthesis of key, hepatic gluconeogenic enzymes (Weber, Singhal & Srivastava, 1965b; Weber, Singhal, Stamm & Srivastava, 1965; Singhal & Weber, 1965).

In the course of recent experiments designed to demonstrate the *direct* effect of steroids (injected via the portal vein) on the activities of these gluconeogenic enzymes in the livers of dogs anaesthetised with pentobarbitone, we found there was no change in these enzyme activities over a 6 hr period. This observation was surprising in view of previous data demonstrating rapid increases in both enzymes after intraperitoneal administration of glucocorticoid hormones in rats (Weber & Singhal, 1964a; Weber & Singhal, 1964b). From these observations, it appeared possible that barbiturate anaesthesia may have interfered with glucocorticoid-induced enzyme synthesis. To test this possibility, experiments were designed in which triamcinolone-induced enzyme synthesis was studied in rats in the absence and presence of pentobarbitone. The results obtained show that pentobarbitone treatment of rats injected with triamcinolone largely prevented the increases in hepatic glucose 6-phosphatase and fructose 1,6-diphosphatase activities.

Male Wistar rats weighing 100–120 g and maintained on Master Laboratory Chow and water *ad libitum* were divided into the following 4 groups: (1) control rats injected with saline; (2) animals injected with pentobarbitone; (3) and (4) triamcinolone-treated rats without and with pentobarbitone administration. Triamcinolone (1 mg/100 g) was injected intraperitoneally every 24 hr for 3 days. Pentobarbitone (1·5 mg/100 g) was also injected intraperitoneally at 12 hr intervals during the 3 day period. In addition, a final injection of pentobarbitone (1·5 mg/100 g) was given 2 hr before the rats were killed. Rats in all groups were killed on the morning of the 4th day. Activities of liver glucose 6-phosphatase and fructose 1,6-diphosphatase were assayed in homogenates and supernatant fluids respectively, according to procedures previously described (Weber, Singhal & Stamm, 1963; Weber & Singhal, 1964b). Protein was estimated using the method of Lowry, Rosebrough, Farr & Randall (1951) and blood sugar determinations were made by the method referred to by Weber & Singhal (1964c) Enzyme activities are expressed as total available activity (Weber & Singhal, 1964a, b) and calculated as μmoles of substrate metabolised per g of liver $\times$ liver to body weight ratio $\times$ 100. The data were subjected to statistical evaluation as described previously (Weber & Singhal, 1964a; Singhal & Valadares, 1966).

Table 1 summarises the effect of pentobarbitone administration on hepatic glucose 6-phosphatase, fructose 1,6-diphosphatase, total protein and blood sugar level in rats treated with triamcinolone for 3 days. Treatment with pentobarbitone alone was without effect on the parameters studied, since values obtained after its administration were similar to those of saline-injected controls. In triamcinolone treated rats, hepatic glucose 6-phosphatase was increased to 205% and fructose 1,6-diphosphatase to 199% of controls. However, in rats treated concurrently with triamcinolone and pentobarbitone, hepatic glucose 6-phosphatase and fructose 1,6-diphosphatase increased to only 119% and 136%

TABLE 1. EFFECT OF INTRAPERITONEAL PENTOBARBITONE ON TRIAMCINOLONE-INDUCED SYNTHESIS OF HEPATIC GLUCONEOGENIC ENZYMES IN WISTAR RATS

Parameter examined	Control	Pentobarbitone	Triamcinolone-treated rats	
			Without pentobarbitone	With pentobarbitone
Glucose 6-phosphatase ..	5187 ± 86 (100)	4928 ± 49 95	10,639 ± 638 205* (100)	6190 ± 536 119; 58†
Fructose 1,6-diphosphatase	2245 ± 49 (100)	2335 ± 104 104	4452 ± 292 199* (100)	3049* ± 300 136; 69†
Total protein	303 ± 18 (100)	342 ± 31 113	440 ± 15 145* (100)	401 ± 14 132*; 9
Blood sugar (mg/100 ml) ..	107 ± 3 (100)	136 ± 10 127	145 ± 11 136* (100)	132 ± 6 123*; 91

Means ± s.e. represent 4 or more animals in each group. Enzyme activities are calculated as μmoles of substrate metabolised per g of liver × liver to body weight ratio × 100. Total protein was estimated in the supernatant fluid and expressed as mg of protein per g of liver × liver to body weight ratio × 100. Data are also given in percentages taking the values of control rats as well as for triamcinolone-injected animals without pentobarbitone administration as 100% (in parentheses).

* Statistically significant difference as compared to the values of control rats ($P = < 0.05$).
† Statistically significant difference as compared to the values of triamcinolone-treated rats without pentobarbitone administration ($P = < 0.05$).

respectively. The increases observed in total protein content and blood sugar leve after triamcinolone treatment were not significantly affected by pentobarbitone.

The possibility that mild generalised sedation produced by pentobarbitone (1·5 mg/100 g every 12 hr) may have been responsible for the observed effects cannot be ruled out; at present we are not certain whether the observed interference in triamcinolone-induced enzyme synthesis can be achieved by other agents with known central depressant effects. It should also be emphasised that although the animals treated with pentobarbitone displayed some decrease in motor activity, their food intake appeared to be normal. It is likely that the observed inhibition of glucocorticoid-induced enzyme synthesis by pentobarbitone may have been due either to a stimulation of a system necessary for inactivation of the steroid hormone or the effects of a direct interaction between triamcinolone and the barbiturate.

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Biochemical effects of some newer salicylic acid congeners

SIR,—The exact mechanism of anti-inflammatory activity of drugs is not well understood. The anti-inflammatory agents like salicylates, butazolidine, resorcylic acid, hydrocortisone, glycyrrhetic acid and imipramine have been shown to inhibit aminotransferases (Moses & Smith, 1961; Huggins, Bryant & Smith, 1961; Huggins, Smith & Moses, 1961; Tangri, Seth, Parmar & Bhargava, 1965; Tangri, Saxena, Seth & Bhargava, 1966), uncouple oxidative phosphorylation (Whitehouse & Haslam, 1962) and stimulate ATP phosphohydrolase activity (Whitehouse & Haslam, 1962; Tangri & others, 1965, 1966); all of these actions may be related to their anti-inflammatory property. Some newer salicylic acid congeners like 2,4-diacetoxybenzoic acid, *m*-cresotinic acid and 5-ethyl-2-hydroxybenzoic acid were reported to have potent anti-inflammatory activity (Tangri & Bhargava, 1964). The effect of these agents on serum aminotransferases and tissue ATP phosphohydrolase has been examined to correlate their biochemical effects with their anti-inflammatory activity.

Enzyme assays were made both in normal and arthritic albino rats with or without drug treatment. Serum for the estimation of aminotransferase activity was obtained from the blood of decapitated rats. The livers were removed immediately and pooled for the estimation of ATP phosphohydrolase (EC. No. 3.6.1.4.) activity. Serum L-aspartate: 2-oxoglutarate aminotransferase (EC. No. 2.6.1.1, aspartate aminotransferase) and serum L-alanine: 2-oxoglutarate aminotransferase (EC. No. 2.6.1.2, alanine aminotransferase) were assayed by the method of Reitman & Frankel (1957). One unit of enzyme activity was the change in the extinction of 0·001/min/ml of serum, which was measured in a Bausch & Lomb Spectronic '20' colorimeter at 505 mμ. ATP phosphohydrolase activity was estimated in 10% (w/v) pooled liver homogenate prepared in 0·25 M sucrose. The reaction mixture consisted of 0·05 M Tris pH 8·0, 1 mM ATP and 0·1 ml of 10% tissue homogenate in a final volume of 2 ml. Release of P_i (inorganic phosphorus) from ATP was measured according to Fiske & Subbarow (1925). The release of 1 μM of P_i/100 mg of tissue in 15 min at 37° was considered as one unit of enzyme activity.

The arthritis in the ankle joints of albino rats (100–110 g) was produced by injecting 0·1 ml of 2% (v/v) formaldehyde subcutaneously under the plantar aponeurosis according to Brownlee (1950). The animals were treated with daily intraperitoneal injections of 2,4-diacetoxybenzoic acid, *m*-cresotinic acid, 5-ethyl-2-hydroxybenzoic acid (2 mg/100 g bodyweight) and hydrocortisone (0·5/100 g bodyweight) for 10 consecutive days.

The results are in Tables 1 and 2. The newer salicylic acid congeners inhibited the normal alanine aminotransferase but did not significantly alter the normal aspartate aminotransferase. A greater sensitivity of alanine aminotransferase to anti-inflammatory agents (viz. salicylates, glycyrrhetic acid, hydrocortisone and imipramine) compared to aspartate aminotransferase has been reported (Huggins & others, 1961a, b; Tangri & others, 1965, 1966). Furthermore, the inflammatory reaction increased the levels of both serum aminotransferases but particularly aspartate aminotransferase. This increase in the enzymic activities due to inflammation was prevented by the salicylic acid congeners to the same degree as hydrocortisone. Other anti-inflammatory agents, glycyrrhetic acid, methyl glycyrrhetic acid and imipramine, also prevented the rise in the serum aminotransferases caused by inflammation (Tangri & others 1965, 1966).

Since the anti-inflammatory agents significantly reduced the enhanced serum aspartate aminotransferase level induced by the inflammatory stimulus and since they failed to alter the normal aspartate aminotransferase activity, it may be

TABLE 1. EFFECT OF HYDROCORTISONE, 2,4-DIACETOXYBENZOIC ACID, *m*-CRESOTINIC ACID AND 5-ETHYL-2-HYDROXYBENZOIC ACID ON SERUM ASPARTATE AMINOTRANSFERASE AND ALANINE AMINOTRANSFERASE IN NORMAL AND ARTHRITIC RATS.
(One unit of enzyme activity is the change in extinction of 0·001/min/ml of serum)

		Control	Hydrocortisone*	2,4-Diacetoxybenzoic acid	*m*-Cresotinic acid	5-Ethyl-2-hydroxybenzoic acid
Serum aspartate aminotransferase	Normal	28·27 ± 1·19	28·1 ± 0·68 (P = 0·9)	31·2 ± 0·63 (P = 0·2)	28·0 ± 1·15 (P = 0·9)	29·8 ± 0·63 (P = 0·5)
	Arthritic	50·0 ± 1·1	28·6 ± 1·31 (P = 0·001)	39·8 ± 0·5 (P = 0·001)	31·6 ± 0·6 (P = 0·001)	37·2 ± 0·5 (P = 0·001)
	Percent increase in inflammation	76·7	1·77	27·5	13·8	25·0
Serum alanine aminotransferase	Normal	32·13 ± 1·5	22·5 ± 1·25 (P = 0·001)	23·8 ± 1·4 (P = 0·02–0·01)	25·2 ± 0·8 (P = 0·05–0·02)	28·0 ± 0·8 (P = 0·2)
	Percent decrease with drug	—	30·0	26·0	21·4	15·7
	Arthritic	39·8 ± 1·7	25·7 ± 1·56 (P = 0·001)	20·0 ± 0·9 (P = 0·001)	25·0 ± 0·6 (P = 0·001)	26·8 ± 0·8 (P = 0·001)
	Percent decrease with drug	—	39·9	49·9	37·9	32·6

* Data reported by Tangri & others (1965).

TABLE 2. EFFECT OF HYDROCORTISONE, 2,4-DIACETOXYBENZOIC ACID, *m*-CRESOTINIC ACID AND 5-ETHYL-2-HYDROXYBENZOIC ACID ON THE ATP PHOSPHOHYDROLASE ACTIVITY IN THE POOLED LIVER HOMOGENATES OBTAINED FROM NORMAL AND ARTHRITIC RATS

		Control	Hydrocortisone*	2,4-diacetoxybenzoic acid	*m*-Cresotinic acid	5-Ethyl-2-hydroxybenzoic acid
Liver ATP phosphohydrolase	Normal	11·62	16·96	16·09	15·21	15·21
	% increase with drug	—	46·1	38·4	30·9	30·9
	Arthritic	11·62	16·99	16·99	16·09	16·99
	% increase with drug	—	46·2	46·2	38·4	46·2

* Data reported by Tangri & others (1965).

implied that the extra aspartate aminotransferase activity caused by inflammation is more sensitive to these drugs and that a correlation for anti-inflammatory action and reduction in enhanced aspartate aminotransferase activity exists. It may well be that there is an enzyme or isoenzyme differing from the ordinary circulating aspartate aminotransferase. On the contrary, no correlation can be made for the inhibition of enhanced alanine aminotransferase activity and the inhibitory effects of the agents since they inhibited both enhanced and normal serum enzyme activity.

Salicylic acid congeners were also found to stimulate the liver ATP phosphohydrolase activity both in the normal and arthritic rats. The anti-inflammatory

activity of these agents is not dependent upon the increased ATP phosphohydrolase, since the inflammatory reaction *per se* did not influence the hepatic ATP phosphohydrolase activity.

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Potentiation of the anticonvulsant action of acetazolamide

SIR,—Certain α- or β-adrenergic blocking agents antagonize the anticonvulsant action of acetazolamide (Rudzik & Mennear, 1966a), but dichloroisoprenaline enhances it. Since the anticonvulsant action of acetazolamide is mediated through a catecholamine mechanism (Gray, Rauh & Shanahan, 1963; Rudzik & Mennear, 1966b), the enhancement of acetazolamide by dichloroisoprenaline may be related to its ability to produce adrenergic stimulation before blockade. We now report the effects of several central nervous system stimulants as well as amine-releasing agents on the anticonvulsant action of acetazolamide.

Male albino mice (Harlan Industries), 18–26 g were dosed with the various agents under investigation 30 min before the intraperitoneal injection of acetazolamide. The ED 50 value (Litchfield & Wilcoxon, 1949) for the anticonvulsant effect of acetazolamide was determined 30 min after the injection of acetazolamide. Maximal electroshock siezures were given (Swinyard, Brown & Goodman, 1952) and the criterion for protection against the seizure was abolition of hind leg extension. The results are in Table 1.

As reported earlier (Rudzik & Mennear, 1966b) dichloroisoprenaline potentiated the anticonvulsant effect of acetazolamide while MJ-1999 [4′-(1-hydroxy-2-isopropylamino) methanesulphonanilide] antagonized it. Dichloroisoprenaline has also been reported to produce a stimulant effect upon the grossly observed behaviour of rats (Randrup, Munkvad & Udsen, 1963), to antagonize reserpine-induced ptosis in mice (Aceto & Harris, 1963) and to enhance the lethal effect of amphetamine in aggregated mice (Mennear & Rudzik, 1965). Intrinsic (adrenergic stimulatory) activity has not been reported for MJ-1999 (Dugan & Lish, 1964).

Since amine-depleting agents have been reported to antagonize the action of

TABLE 1. EFFECTS OF SEVERAL PHARMACOLOGIC AGENTS ON THE ANTICONVULSANT ACTION OF ACETAZOLAMIDE IN MICE

Treatment	Intraperitoneal dose (mg/kg)	Change from control ED50
Dichloroisoprenaline ..	10	−40*
MJ-1999	30	+192*
Metaraminol	10	−77*
(+)-Amphetamine ..	2·0	−52*
Ephedrine	5·0	−70*
Caffeine	5·0	−58*
Imipramine	5·0	−51*
Desipramine	5·0	−40*
Tranylcypromine ..	10	−52*
Cocaine	5·0	−32

* Significantly different from control value ($P > 0.05$).

acetazolamide (Rudzik & Mennear, 1966a) it was interesting to find that metaraminol, when administered 30 min before acetazolamide, produced potentiation. This, however, may be a reflection of the release of catecholamines by metaraminol. In support of this hypothesis is our finding that after 4 hr of pretreatment with metaraminol the anticonvulsant action of acetazolamide is significantly antagonized (control ED 50 23 mg/kg *vs.* treated ED 50 74 mg/kg).

The potentiation of acetazolamide by amphetamine, imipramine, desipramine and tranylcypromine, each of which is known to influence the disposition of catecholamines, lends support to the hypothesis that acetazolamide produces its anticonvulsant action through a catecholamine mechanism. The failure of cocaine to produce significant potentiation of acetazolamide may be related to its biphasic effect on tissue levels of catecholamines (Potter, Axelrod & Kopin, 1962).

A number of pharmacologic agents were found to be without effect on the anticonvulsant action of acetazolamide. These agents include: tyramine, mepyramine, morphine, and SKF-6890 [2-(2,6-dimethylphenoxy)ethyltrimethylammonium bromide].

It seems that a catecholamine mechanism may be involved in the potentiation of the anticonvulsant action of acetazolamide as well as its anticonvulsant effect *per se*.

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Failure of antihistamine to protect against histamine-induced duodenal ulceration in the guinea-pig

SIR,—Previously it was demonstrated that the antihistamine drug, mepyramine maleate, reduces the incidence and greatly reduces the severity of gastric ulceration induced by histamine (Watt & Eagleton, 1964). We have since elaborated a method whereby histamine ulceration can be limited entirely to the duodenum (Eagleton & Watt, 1966). It was of interest therefore, to determine the effect of mepyramine on the production of such lesions in the guinea-pig.

Male albino guinea-pigs of 550 g were deprived of food for 12 hr before the start of the experiment and wore Perspex collars to prevent coprophagy. Water was supplied *ad lib.*

Duodenal ulceration was induced by the repeated intramuscular injection of histamine acid phosphate in aqueous solution (1 mg/ml). The injections (0·25 mg/kg) were given at 30 min intervals over a period of 3½ hr. Animals which received mepyramine maleate were given 10 mg/kg intramuscularly ½ hr before and again 2 hr after the first injection of histamine.

All animals were killed by a sharp blow on the head 4 hr after the first injection of histamine. The gastric juices were removed, their volumes measured, and total acid concentrations measured by titration against 0·04 N sodium hydroxide using phenolphthalein as indicator. The stomach and upper small intestine were distended with formol-saline and viewed by transillumination. The extent of damage was expressed in terms of the length of small intestine involved.

The incidence of histamine-induced duodenal ulceration in the control group and in the group treated with mepyramine maleate are shown in Table 1. In both groups the incidence of ulceration was 100%. There was no statistically significant difference in respect of the extent of involvement of the small intestine ($P > 0.70$) or in relation to juice volume ($P > 0.50$) and acid concentration ($P > 0.30$) of the juices recovered at the end of the experiments.

TABLE 1. THE INCIDENCE AND EXTENT OF HISTAMINE-INDUCED DUODENAL ULCERATION IN MALE ADULT GUINEA-PIGS WITH AND WITHOUT ANTIHISTAMINE (2 × 10 MG/KG I.M.) (MEANS ± ONE S.D.)

	% with duodenal ulcers	Length of small intestine involved (cm)	Gastric juice at end of experiment	
			Volume (ml)	Total acid (m-equiv./litre)
With mepyramine (10 animals) ..	100	7·9 ± 1·8	5·6 ± 4·3	78·6 ± 14·7
Without mepyramine (10 animals) ..	100	8·7 ± 1·9	6·8 ± 4·8	68·8 ± 39·8

The failure of the antihistamine, mepyramine, to prevent histamine-induced duodenal ulceration is in striking contrast to the protective action of mepyramine against ulceration of the stomach induced by the same ulcerogenic agent. The dose of mepyramine used in this study was the same as that in the previous study in which protection was demonstrated against gastric ulceration caused by much larger doses of histamine (Watt & Eagleton, 1964). It is unlikely therefore that the failure to prevent duodenal lesions is due to inadequate dosage of antihistamine. It is of some interest that in the above experiments there was no suggestion that antihistamine materially altered the acidity or volume of gastric juice recovered at the end of the 4 hr period during which ulcers were forming.

The observation that an antihistamine drug interrupts the process of ulceration

in the stomach but not in the duodenum is interpreted as evidence that the mechanism whereby histamine causes gastric ulceration is different from that whereby histamine causes duodenal ulceration.

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A comparison of fluphenazine and chlorpromazine on critical flicker fusion frequency

SIR,—Besser, Duncan & Quilliam (1966) showed that chlorpromazine 25 and 50 mg depressed the auditory flutter fusion threshold at 90 and 180 min after administration, but neither 2 nor 4 mg of perphenazine altered it. It is also true that piperazine phenothiazine derivatives have less sedative effects than aliphatic derivatives.

Visual critical flicker frequency is a similar test of central nervous function. In a double-blind experiment, identical tablets of fluphenazine 1 mg, chlorpromazine 25 mg and a placebo were administered in random order to 5 young subjects, of either sex, in a Latin square design and with an interval between administrations of not less than 3 days. The critical flicker frequency was measured at 0, 2, 4 and 7 hr (Turner 1965, Smart & Turner, 1966) which involved exposing the subjects to intermittent light at 25 and 50 c/sec, for 1 min before measuring the critical flicker frequency.

Chlorpromazine produced a fall in the mean critical flicker frequency threshold between 0 and 4 hr compared with the placebo ($P < 0.02$) but the change in threshold after fluphenazine was not significantly different from that after the placebo. Between 4 and 7 hr the threshold after chlorpromazine rose towards the resting level but was still depressed ($P < 0.05$). The difference between the effects of chlorpromazine and the other two treatments was significant ($P < 0.01$). None of the treatments influenced the adapting effect of light at 20 and 50 c/sec on the parameter, which is a stable phenomenon (Turner, Patterson & Smart, 1966).

It appears, therefore, that in this sensitive test of visual discrimination, the aliphatic phenothiazine derivitive chlorpromazine has a significant depressant action compared with the piperazine derivative fluphenazine.

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Interaction of gases with monolayers

SIR,—Two apparently contradictory reports have been made, by Clements & Wilson (1962) and by Evans, Hamilton, Kuenzig & Peltier (1966), about the effects of gaseous anaesthetic agents on monomolecular films. Whereas Clements & Wilson (1962) showed that halothane had a significant effect on the surface pressure of a number of monomolecular films, Evans & others (1966) concluded that halothane did not alter significantly the surface pressure of dipalmitoyl lecithin films. Clements & Wilson, maintained their films at fixed areas and low surface pressures, whilst Evans & others varied their film areas and pressures over a wide range by use of a variable area trough. These latter films were subjected to repeated rapid compression-expansion cycles during which pressures of almost 70 dynes/cm, with average standard deviations of more than 2 dynes/cm, were observed. Based on our experience of the effects of gases on monolayers, the apparent differences in the results are probably directly traceable to basic differences in procedures and techniques.

Monomolecular films have been shown to be useful as biological models in the study of membrane interactions with a wide variety of materials in solution (Schulman & Rideal, 1937). It is reasonable to expect that such models could be applied to the study of membrane-gas interactions.

In recent work on the effect of gaseous air pollutants on monolayers we have established the need for rigid experimental standardization of the films to be used in gas-film studies. Without such standardization, meaningless and often misleading conclusions could be reached. A precise knowledge of the film properties in the absence as well as in the presence of the gases is required. For example, the rate of film compression, temperature, and history of the film markedly influence the surface pressure-surface area (π-A) isotherms of stearic acid monolayers (Rabinovitch, Robertson & Mason, 1960). Whereas the collapse pressure of stearic acid films has been reported to be as high as 62 dynes/cm using rapid compression methods (Dervichian, 1937), with very slow manual compression rates these films yield collapse pressures of about 15 dynes/cm. At pressures above 15 dynes/cm, the surface pressure values become significantly time dependent. More important, when the π-A isotherms were obtained for films subjected to repeated compressions and expansions, marked changes occurred in the shapes of the isotherms, especially in condensed regions. Similar effects would be expected for phospholipid films.

Furthermore, reproducibility of surface pressure values on successive highly condensed films, can produce serious errors. We have found that an error of 1% in the amount of spreading solution added to the subphase surface can easily produce errors of more than 10 dynes/cm at areas near the limiting area of the film molecules. Such large errors can easily obscure the small effects expected for low concentrations of gases with monolayers. Measurements of surface pressures on a single film, before and after exposure to the gas, would eliminate this source of error.

It has been shown that when interactions with the film are relatively weak, the interacting material may be squeezed out of the film at high pressures (Cockbain & Schulman, 1939). It would be expected that in most instances the gas-film interactions would be weak, involving only van der Waals' forces. Thus a gas which normally interacts with a film under expanded conditions might exhibit little or no interaction with a condensed film or with an expanded film having a history of compression beyond the "equilibrium" collapse pressure.

We therefore feel that more meaningful information would be obtained for the interactions of gases with film molecules when these are made on a single

film whose π-A characteristics have first been established in the absence of the gases. The rates of compression should be slow enough to allow the molecules in the monolayer to maintain "equilibrium" orientations at all areas at which measurements are made. Furthermore, the film should never be compressed beyond the collapse pressure when further expansion-compression cycling of the film is desired.

We have found that when these experimental conditions are adhered to, effects of gases on films that result in small surface pressure changes can be easily detected.

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The effect of bradykinin and anti-inflammatory agents on isolated arteries

SIR,—Bradykinin constricts isolated perfused arteries from guinea-pig lung (Moog & Fischer, 1964) and from rabbit lung (Hauge, Lunde & Waaler, 1964). This vasoconstrictor effect is abolished by phenylbutazone (Klupp & Konzett, 1965), acetylsalicylic acid (Greeff & Moog, 1964), and flufenamic and mefenamic acids (Bauer, Gmeiner & Konzett, 1965) although the antagonism has been shown to be non-specific (Hauge, Lunde & Waaler, 1966). We have now examined the responses of other arteries to these drugs by the method of de la Lande & Rand (1965). Bradykinin and its antagonists were injected through the cannula into the perfusion fluid (McEwen's solution, 1956) which supplied the isolated artery preparation. Histamine, 5-hydroxytryptamine, acetylcholine, noradrenaline and kallidin were our standards of comparison. On occasions, the upper ends of the vessels were stimulated electrically (pulse width 1 msec, strength 10–20 V, frequency 1–20/sec) using bipolar platinum electrodes.

In our hands, also, bradykinin (1–10 μg) constricted the isolated pulmonary artery of the rabbit and the anti-inflammatory agents (sodium phenylbutazone, sodium mefenamate, sodium flufenamate, sodium meclofenamate and calcium acetylsalicylate) antagonised the response when administered by slow infusion (0·1–2·5 mg/min). The same concentrations of antagonists also antagonised the vasoconstrictor responses to histamine (0·1–0·5 μg), 5-hydroxytryptamine (5-HT) (0·1–0·5 μg), acetylcholine (0·2–10 μg) noradrenaline (0·1–0·5 μg) and kallidin (0·2–2 μg), and so it was proved that the antagonism was non-specific. The effects of electrical stimulation were also greatly reduced by the anti-inflammatory agents. In some experiments, tachyphylaxis to repeated doses of bradykinin was noted.

Bradykinin had little or no effect on the ear, femoral, mesenteric, brachial and carotid arteries of the rabbit unless these had been constricted during electrical stimulation. Under these circumstances, bradykinin (1–10 μg), kallidin (0·5–5 μg) and acetylcholine (0·1–1 μg) were all vasodilator in action (see Fig. 1). On the other hand, histamine, 5-HT and noradrenaline were always vasoconstrictor, the contractions they produced being superimposed on those already present when electrical stimulation was applied. Doses of atropine (0·01–0·2 μg) which completely abolished the response to acetylcholine did not modify the responses to bradykinin and kallidin. The ear and mesenteric vessels were the most sensitive of the rabbit arteries responding with dilatation to bradykinin, and tachyphylaxis was not found in any of these preparations. The anti-inflammatory agents did not modify the vasodilator action of bradykinin.

All the seven corresponding arteries of the guinea-pig were constricted by bradykinin (0·1–10 μg), the brachial, femoral and carotid vessels being the most sensitive (see Figs 1 and 2). Histamine (5–20 μg), 5-HT (5–20 μg) and noradrenaline (0·4–10 μg) also constricted the vessels and often markedly potentiated the subsequent bradykinin effect. Doses of mepyramine, bromolysergic acid diethylamide and phentolamine which blocked the responses to histamine, 5-hydroxytryptamine or noradrenaline did not affect those of bradykinin.

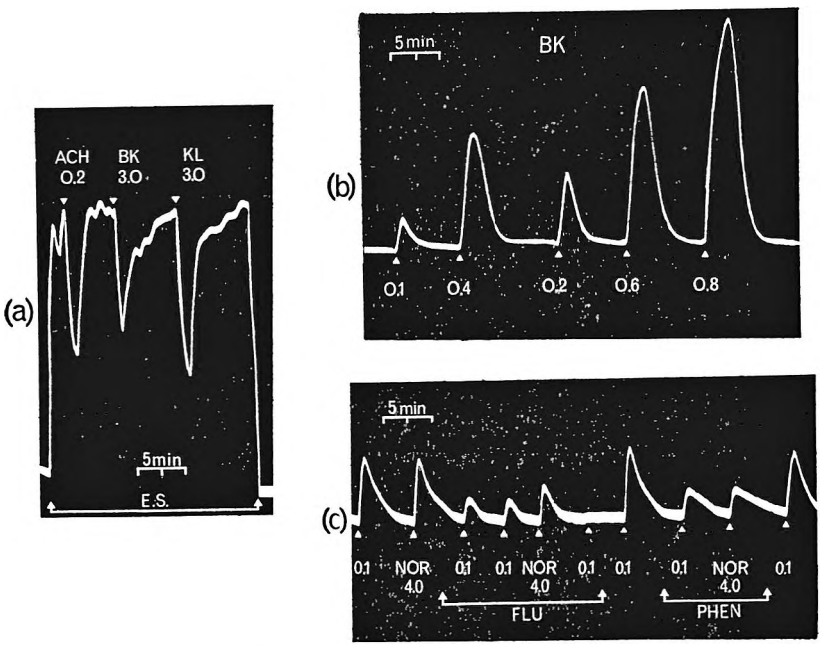

FIG. 1 (a). Effect of bradykinin (Bk, μg) on the isolated rabbit ear artery. Continuous stimulation (E.S.) shown between the arrows. The effects of acetylcholine (ACH, 0·2 μg) and kallidin (KL, 3 μg) are also shown.

(b). Responses of the isolated artery from guinea-pig leg to various doses of bradykinin. Note that bradykinin relaxes the rabbit ear artery but constricts the guinea-pig femoral artery.

(c). Effects of the anti-inflammatory drugs, sodium flufenamate (FLU, 0·64 mg/min) and sodium phenylbutazone (PHEN, 0·64 mg/min), on the responses of the guinea-pig mesenteric artery to bradykinin (0·1 μg) and noradrenaline (NOR, 4 μg).

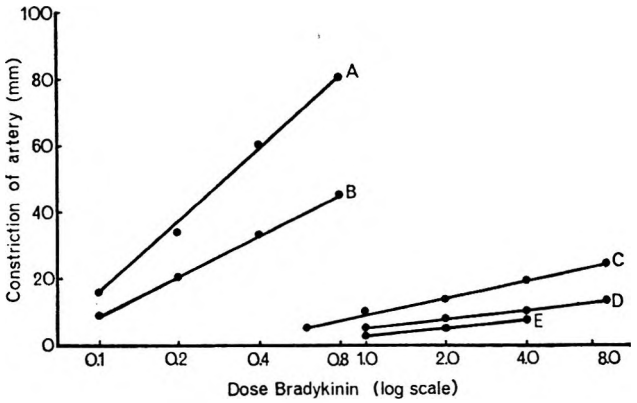

FIG 2. Log-dose response curves for bradykinin (μg) using brachial (A), femoral (B), carotid (C), mesenteric (D) and renal (E) arteries of the guinea-pig.

However, as with rabbit arteries, infusions of the anti-inflammatory agents which blocked the response to bradykinin also blocked responses to the other constrictor agents, and when the infusions ceased, recovery of all the vasoconstrictor actions was immediate (see Fig. 1). On many guinea-pig artery preparations, bradykinin was often 10–50 times more active than noradrenaline. Except on the pulmonary artery, tachyphylaxis to repeated doses of bradykinin was not found.

The seven corresponding arteries of the rat responded to bradykinin like those of the rabbit, only the pulmonary artery constricting. Kallidin was usually about twice as active as bradykinin in these preparations.

It is evident that the vasoconstrictor action of bradykinin on isolated mammalian arteries is antagonised in a non-specific manner by these anti-inflammatory agents. All artery preparations from guinea-pigs were constricted by bradykinin but only those of the pulmonary vessels of the rat and rabbit responded in this way.

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