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Potentiometric determination of sodium using a sodium ion responsive glass electrode

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A study to assess the feasibility of using a sodium ion responsive glass electrode in conjunction with a saturated calomel reference electrode to measure the sodium ion concentration of a wide range of electrolyte solutions used in clinical medicine has shown that the method is capable of giving results which are within acceptable limits. Direct measurement of solutions containing sodium chloride is possible by reference to a calibration based on the potential produced by the electrode pair as a function of pNa^+ defined as $-\log_{10}$ sodium ion concentration. For the measurement of the sodium content of solutions of sodium salts of weak acids and mixed solutions of electrolytes and dextrose it is necessary to use a calibration carried out in a buffer system (0·5M triethanolamine + hydrochloric acid to pH 7) and to dilute the preparations with buffer before measurement. It is also necessary to buffer dextrose and sodium chloride injection before measurement due to the effect of decomposition of dextrose during sterilization causing a shift in pH which must be corrected before making a determination. The advantages of the potentiometric method over conventional flame photometric and titrimetric methods are discussed and a brief review of the literature given, to indicate the applications of specific ion electrodes.

The early studies of Eisenman, Rudin & Casby (1957) using glass membranes highly selective to sodium ions have resulted in these electrodes being commercially available. Mattock (1962; 1967) has described the practical aspects and properties of these electrodes. Specific ion electrodes sensitive to other cations, K^+ and Ca^{2+}, and anions of the halide series are also available.

Applications of specific ion electrodes in analysis (Jacobson, 1968) can be extended to measure very low sodium concentrations as described by Hawthorn & Ray (1968) who measured sodium ion concentrations in water in the range of 0·004 to 25 ppm of sodium. These electrodes have also been used in solubilization studies involving micellar sodium dodecyl sulphate solution (Pearson & Lawrence, 1967). *In vitro* measurements have been made on a wide range of biological fluids, including blood, cerebrospinal fluid, sweat, urine, bile and brain extracts; work carried out *in vivo* has included the continuous measurement of plasma sodium levels of rabbit and dog (Friedman, Jamieson & others, 1958) and the intracellular measurement of sodium and potassium activities in the muscle cells of crab and lobster (Hinke 1959). Recent reviews of work in these and related fields have been given by Moore (1968) and Carr (1968).

We have used a sodium ion responsive glass electrode in conjunction with a saturated calomel reference cell for the measurement of the sodium content of a wide range of

electrolyte solutions. For some solutions the results are compared with those obtained using alternative conventional assay procedures. For solutions with sodium present only as chloride, direct chloride ion titration using standard silver nitrate has been used, and where sodium salts other than, or in addition to, chloride are present, flame photometry has been adopted.

EXPERIMENTAL

Reagents. For calibration and non-sterilized solutions, dry analytical grade chemicals were used. All sterilized infusions, injections and dialysis solutions tested were prepared with B.P. quality starting materials. The buffer solution was prepared using reagent grade materials.

Apparatus and operating conditions. The electrode pair consisted of a sodium ion responsive glass electrode [Electronic Instruments Ltd., (E.I.L.), Type GEA 33] in conjunction with a calomel reference electrode employing a saturated potassium chloride salt bridge (E.I.L. Type RJ23). Trial experiments were undertaken using an E.I.L. Model 46A pH meter for the measurement of potential output at a controlled temperature of 25 $\pm$ 0·1°; most measurements were made using a Pye Model 79 pH meter on solutions at room temperature within the range 21–28°. The electrodes were always kept moist. The sodium ion responsive electrode was stored in 0·1M sodium chloride and the calomel reference electrode in saturated potassium chloride solution between series of measurements. The electrodes when in use were lightly wiped with a tissue after a measurement and rinsed with the next solution before inserting the electrode pair into the bulk sample volume. Values of potential were recorded after approximately 3 min, although, owing to a rapid response time, equilibration was often complete before this time.

Procedure. Calibration was carried out before and after a series of measurements. For the initial calibration, standard solutions of 0·5, 0·1 and 0·01M sodium chloride in distilled water were used and a calibration graph plotted of electrode response in mV against pNa^+ ($-\log_{10}$ molar sodium ion concentration). Direct measurements of potential were made on 0·45% w/v, 0·76% w/v and 0·9% w/v sodium chloride test solutions without dilution, and on 28·8% w/v sodium chloride solution after diluting 1 in 32.

A second calibration graph was plotted of electrode response against pNa^+, using standard solutions of sodium chloride 0·1, 0·05, 0·01 and 0·001M in buffer solution (0·5M triethanolamine + hydrochloric acid to pH 7). Test solutions containing sodium salts of weak acids, mixed solutions of electrolytes with and without dextrose, and solutions of sodium chloride and dextrose were suitably diluted with the buffer solution before measurement. For dextrose 4·3% w/v with sodium chloride 0·18% w/v injection, four samples were diluted 1 in 10 with buffer, and the remainder by a factor of 5. Dilutions of the other solutions are given in Tables 2 and 4.

RESULTS

Calibration

Fig. 1 shows a full range calibration graph obtained at 25° for sodium chloride solutions made up using distilled water. The electrode response in mV is expressed both as a function of molar sodium ion concentration and activity, using literature values for the mean molal ionic activity coefficients (Scatchard & Prentiss, 1933). Ignoring the small error involved in using molal activity coefficients it can be seen from

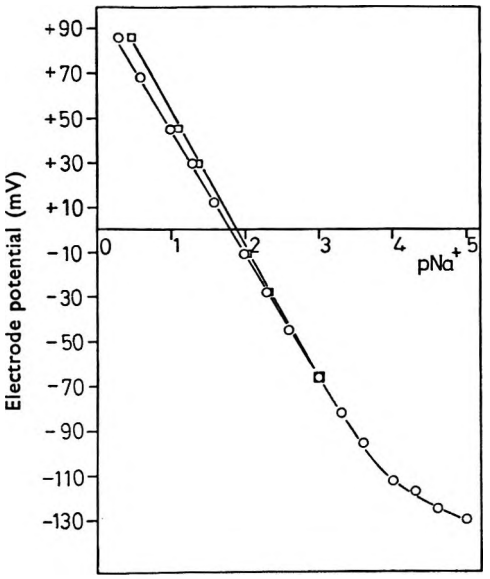

FIG. 1. Full range calibration graph based on unbuffered sodium chloride solutions showing potential of electrode pair (mV) as a function of pNa^+ defined as $-\log_{10}$ activity Na^+ (□) and $-\log_{10}$ concentration Na^+ (○).

Fig. 1 that the electrode response is a linear function of pNa^+ ($= -\log_{10}a_{Na^+}$) down to $pNa^+ = 3$ when deviation occurs. The experimental slope in the linear region for a ten-fold change in activity was 59·0 mV, in good agreement with the theoretical Nernst slope (2·303 RT/F) of 59·2 mV at 25°. Defining pNa^+ in terms of molar sodium ion concentration (i.e. $pNa^+ = -\log_{10}c_{Na^+}$) also results in a linear plot, of slope 56·0 mV in the linear region, and this plot has proved suitable for the direct analysis of sodium chloride solutions. Experience over a period of ten months has shown remarkable consistency in that potential values recorded for any given standard solution do not differ by more than about ±1 mV thus showing negligible long-term drift.

Using the buffer system the calibration plot was linear down to pNa^+ (concentration) = 3·5 with a slope of 59·2 mV. Diluting preparations with buffer before measurement ensured a medium of approximately constant ionic strength and pH, at least 10–20 times as strong as the sodium solution to be measured.

Products containing sodium chloride only

The sodium content of over 250 samples of sodium chloride solution 0·9% w/v (154 m-equiv/litre Na^+) was determined. All the recorded values fell within the range 148–159 m-equiv/litre Na^+ and this may be compared with a range of 147–156 m-equiv/litre Cl^- for the same solutions titrated using standard silver nitrate. Other simple products for which the method proved suitable included 0·45, 0·76 and 28·8% w/v sodium chloride solutions.

Injection of sodium chloride 0·18% *w/v and dextrose* 4·3% *w/v*

Preliminary attempts to analyse samples of this preparation for sodium content by reference to a calibration graph based either on aqueous sodium chloride solutions or sodium chloride in 4·3% w/v dextrose solution produced high results when compared

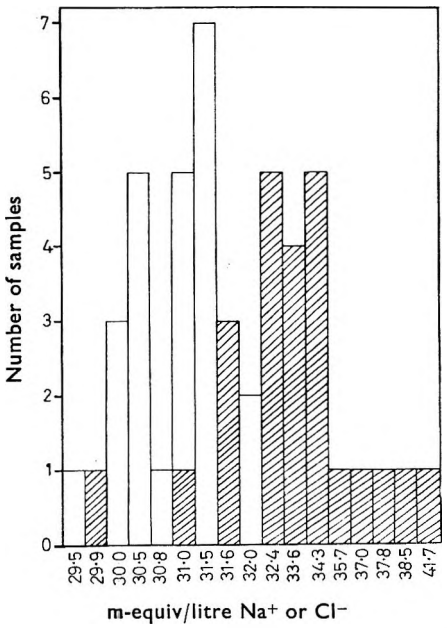

FIG. 2. Frequency histogram showing analytical results (m-equiv/litre Na^+ or Cl^-) for 24 samples of sodium chloride 0·18% w/v and dextrose 4·3% w/v injection as determined by the potentiometric method with reference to a calibration based on unbuffered solutions (shaded blocks) and by titration with standard silver nitrate (open blocks).

with the silver nitrate titration values. The results for 24 samples are given in Fig. 2 which indicates that over half of these are apparently above the 5% error limit by the electrode method, whereas all the preparations are acceptable by the silver nitrate titration method. Further investigation showed that the anhydrous dextrose B.P. used to prepare the solutions contained 0·0063% w/w sodium ion which would not be detected by silver nitrate titration. This would contribute about 0·1 m-equiv/litre Na^+ and is clearly not sufficient to account for the discrepancy.

The pH of solutions containing dextrose is known to decrease on autoclaving due to the production of decomposition products (Hudson & Tarlowski, 1947, Wing, 1960). Since the response of the sodium ion glass electrode is dependent on pH in certain regions, an experiment was undertaken to measure the pH and sodium ion content of solutions autoclaved for different times. A sample of the preparation was subdivided into four portions which were then autoclaved for the time intervals shown in Table 1. Samples were cooled as rapidly as possible after removing from the autoclave and the pH and sodium ion content measured by reference to a calibration graph prepared using unbuffered aqueous sodium chloride solutions. Mattock (1967) has shown that the observed pNa^+ may be expected to decrease as the pH drops below 5 for solutions in the region of 1·5 pNa^+; i.e. the solutions become apparently stronger in sodium ion content. (For stronger solutions e.g. sodium chloride solution 0·9% w/v with pNa^+ 0·81, variations in pH over this region do not affect the response.)

After the officially recommended sterilization time of 30 min, Table 1 shows that the observed sodium ion concentration is already about 3·5% greater than the theoretical value of 31 m-equiv/litre Na^+. Autoclaving times in excess of 30 min or prolonged cooling conditions will rapidly lead to results in excess of 5% of the theoretical value.

Table 1. *The effect of pH changes on the measured sodium ion content of sodium chloride* 0·18% *w/v and dextrose* 4·3% *w/v injection autoclaved for different times*

Autoclave time (min) at 115–116° C	pH	Observed Na^+ (m-equiv/litre)
0	6·1	31·6
15	5·2	31·6
30	4·9	32·1
45	4·5	33·3
60	4·3	35·5

Tc eliminate the effect of pH, calibration and measurements were made in buffer solution. The results of 30 determinations were within the range 30·0 to 31·9 m-equiv/litre Na^+ which are within ±5% limits. Silver nitrate titration results for the same samples gave values in the range 30·0 to 31·5 m-equiv/litre Cl^-.

Sodium salts of weak acids

Preliminary attempts to analyse salt solutions of this type using a calibration graph for aqueous sodium chloride gave results which were far greater than 5% below the theoretical values. However, using the buffer system, improved values were obtained and results for 15 samples are presented in Table 2; some figures are included for samples which were also analysed using standard titrimetric methods.

Table 2. *Analysis of the sodium ion content of sodium salts of weak acids.*

Product	Sample number*	Dilution with Buffer†	m-equiv/litre Na^+ Found‡	Theoretical
Sodium bicarbonate 2·5% w/v	1	1/50	301	298
2·5% w/v	2	1/50	288	
2·5% w/v	3	1/10	313 (305)	
2·5% w/v	4	1/10	309 (298)	
8·4% w/v	5	1/20	1026 (1005)	1000
8·4% w/v	6	1/20	1000 (1010)	
Sodium citrate 3·8% w/v	7	1/100	398	387
3·8% w/v	8	1/100	398	
Sodium lactate $\frac{1}{6}$ M	9	1/25	169	167
$\frac{1}{6}$ M	10	1/25	167	
1 M	11	1/20	1000 (1033)	1000
1 M	12	1/20	1000 (1000)	
1 M	13	1/20	1026 (1043)	
1 M	14	1/20	1000 (1043)	
Sodium thiosulphate 4% w/v	15	1/50	316	322§
4% w/v	16	1/50	316	

* Each sample number refers to a different batch of material.
† 0·5M triethanolamine + hydrochloric acid to pH 7.
‡ Values in brackets refer to results obtained using standard titrimetric methods.
§ Based on $Na_2S_2O_3.5H_2O$.

Mixed electrolyte solutions

The solutions examined showed a wide range of composition and the formulae are shown in Table 3. Results available at the present time for the determination of

Table 3. *Composition (% w/v) of complex electrolyte mixtures*

Component	Compound sodium lactate injection	Darrow's solution	Butler's solution (modified)	Intraperitoneal dialysis solution 1	Intraperitoneal dialysis solution 2	Intraperitoneal dialysis solution 3	Concentrated haemodialysis solution (32·08 ×)	Concentrated haemodialysis solution (25 ×)
Sodium chloride	0·6	0·4	0·06	0·56	0·56	0·56	18·75	14·6
Sodium lactate	0·34	0·36	0·22	0·5	0·5	0·5	—	—
Sodium metabisulphite	—	—	—	0·05	0·05	0·005	—	—
Sodium acetate	—	—	—	—	—	—	7·9	6·2
Potassium chloride	0·04	0·27	0·1	—	—	—	0·36	0·47
Potassium phosphate	—	—	0·05	—	—	—	—	—
Calcium chloride (hyd.)	0·04	—	—	0·039	0·039	0·039	0·532	0·16
Magnesium chloride	—	—	—	0·015	0·015	0·015	0·154	0·12
Dextrose (hyd.)	—	—	5·0	1·5	7·0	1·5	15·4	12·0
Distilled water	to 100 ml	to 100 ml	to 100 ml	to 100 ml	to 100 ml	to 100 ml	to 100 ml	to 100 ml

Intraperitoneal dialysis solution
1 = modified lactate formula, 2 = modified lactate formula, 3 = lactate formula B.P.C. 1968.

Table 4. *Analysis of the sodium content of complex electrolyte mixtures*

Product	Sample number*	Dilution with buffer†	m-equiv/litre Na^+ Found‡	m-equiv/litre Na^+ Theoretical
Compound sodium lactate injection	1	1/5	129	131
	2	1/5	129	
	3	1/5	126	
	4	1/5	129	
	5	1/10	132	
Darrow's solution	6	1/5	115	118
	7	1/5	112	
Intraperitoneal dialysis solution 1	8	1/5	144	141
	9	1/5	144	
	10	1/5	144	
	11	1/5	144	
Intraperitoneal dialysis solution 2	12	1/5	144	141
	13	1/5	141	
	14	1/5	144	
	15	1/5	148	
Intraperitoneal dialysis solution 3	16	1/10	146 (139)	141
	17	1/10	141 (142)	
	18	1/10	138 (139)	
	19	1/10	138 (142)	
Concentrated haemodialysis solution 1	20	1/50	130 (128)	130§
	21	1/50	130 (131)	
	22	1/50	130 (128)	
	23	1/50	130 (128)	
	24	1/50	130 (129)	
	25	1/50	130 (129)	
	26	1/250	132 (130)	
	27	1/250	132 (130)	
	28	1/250	132 (129)	
Concentrated haemodialysis solution 2	29	1/50	131 (132)	130§
	30	1/250	132 (134)	

* Each sample number refers to a different batch of material.
† 0·5M triethanolamine + hydrochloric acid to pH 7.
‡ Values in brackets refer to results obtained using a flame photometer.
§ Theoretical value based on final haemodialysis solution.

sodium ion content are shown in Table 4; results are also given for some of the samples in which the Na^+ content has been measured using flame photometry. Table 4 shows that the electrode method may be considered suitable for application to complex ionic mixtures without the need for detailed information on sodium ion activity coefficients in such systems; more work is currently being undertaken to gain further experience in these solutions.

DISCUSSION

Consideration of the results obtained for the analysis of sodium chloride solutions shows that the potentiometric method gives results which are within the demanded $\pm 5\%$ of the theoretical value. A calibration based on e.m.f. response against sodium chloride concentration in water is satisfactory, although in theoretical terms the electrode responds to sodium ion activity. Measurements made entirely using a buffer system throughout may be also expected to be satisfactory, although the present results indicate that this is not necessary for such simple solutions.

Previous workers have statistically compared the values obtained using a sodium ion responsive glass electrode with those obtained using a flame photometer on the same solutions. Moore & Wilson (1963) working with serum, urine and cerebrospinal fluid showed that the average difference between duplicate photometer measurements was 0·8 m-equiv/litre Na^+, while the average difference in similar electrode measurements was 0·3 m-equiv/litre Na^+; they also found that the electrode variability was less than half that of the flame photometer in each fluid. Annino (1967), in developing a method for the rapid routine measurement of sodium in urine, obtained excellent agreement in a blind comparison of sodium concentrations obtained by flame photometry and a sodium ion responsive glass electrode. Some of the results presented in this paper would seem to confirm these conclusions and although a complete statistical analysis has not yet been made, preliminary observations indicate a favourable comparison between electrode values and those obtained by standard titration techniques.

For solutions of 0·18% w/v sodium chloride and dextrose 4·3% w/v it was found rather unexpectedly that a calibration based on aqueous sodium chloride was not satisfactory. The effect of pH on the pNa^+ readings for these solutions drew attention to the effects of dextrose decomposition on autoclaving (Hudson & Tarlowski, 1947; Webb, Sperandio & Martin, 1958). Decomposition products may affect the sodium ion activity in addition to the direct effect of pH and future applications of the electrode in this area may be indicated. The observed pH range for this preparation is 4·2–5·0 which again emphasises the importance of sterilization conditions.

For the complex electrolyte mixtures, in addition to the possible effects of other ions on sodium ion activity, the decomposition of dextrose during autoclaving has been found to increase in the presence of sodium lactate and potassium phosphate (Griffen & Marie, 1958; Wing, 1960). The satisfactory results obtained by calibration of the electrode system on a pNa^+ (concentration) against e.m.f. basis in buffer would seem to make any reference to the question of sodium ion activity in complex electrolyte mixtures unnecessary. This also holds for the buffer method when applied to sodium salts of weak acids (Table 2) and it is likely that the range of compounds of this type could be greatly extended. For the solutions used in intermittent haemodialysis the sodium content has to be maintained within very narrow limits (Scribner, Fergus & others, 1965). Reference to Table 4 shows that preliminary results obtained for

haemodialysis solutions are well within $\pm 3\%$ of the theoretical value, inferring that the buffer was sufficient to swamp any possible interference effects due to pH changes or other ions. It may however be advantageous to study the electrode behaviour in buffer systems other than that used in the present work and at different dilution ratios.

The present work was undertaken with average pH equipment, the more sensitive E.I.L. instrument discriminating to about $\pm 0{\cdot}5$ mV; for greater precision however it is recommended that a more sensitive instrument is used. Although temperature effects within the quoted range appeared to have negligible effect on the measurements, determinations made on solutions at constant temperature would lead to greater accuracy; controlled temperature conditions would almost certainly be required for use with a sensitive meter.

The commercial development of electrodes sensitive to a wide range of cations and anions is rapidly leading to a new era in chemical analysis and it is suggested that the potentiometric method offers advantages in terms of convenience, ease of manipulation and speed of assay over conventional flame photometric and titrimetric methods. It is therefore anticipated that specific ion electrodes will find many future applications in the pharmaceutical sciences for example in official assay procedures, clinical investigations and general medical technology.

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The *in vivo* and *in vitro* effect of diphenylhydantoin and phenobarbitone on K^+-activated phosphohydrolase and (Na^+, K^+)-activated ATPase in particulate membrane fractions from rat brain

BENT FORMBY

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The effect of diphenylhydantoin and phenobarbitone on a K^+-activated phosphohydrolase, hydrolysing the artificial substrates *p*-nitrophenylphosphate and acetylphosphate, and (Na^+,K^+)-activated ATPase in particulate membrane fractions from rat brain, has been studied. Diphenylhydantoin was given orally over 20 weeks without any effect on these enzymic activities, whereas orally phenobarbitone significantly decreased the enzymic activities in the particulate membrane fractions containing synaptosomes, nerve endings and microsomes. *In vitro*, diphenylhydantoin inhibited the enzymic activities in the synaptosomal membrane fraction, but phenobarbitone did not. Several possibilities for the *in vivo* action of diphenylhydantoin are outlined; these are mainly concerned with a blocking of the passive movement of sodium into the cell. The *in vivo* effect of phenobarbitone is possibly a secondary pharmacological effect interfering with the utilization of ATP either directly or indirectly by a depression of some energy dependent processes such as protein synthesis.

5,5'-Diphenylhydantoin exerts anti-epileptic activity (Toman, 1952), stabilizes membrane-potentials (Esplin, 1957) and lowers the intracellular concentration of sodium of neural tissue (Woodbury, 1955; Pincus & Rawson, 1969). Since there is evidence that the Mg^{2-}-dependent (Na^+,K^+)-activated ATPase is the carrier mechanism involved in the active transport of sodium (Skou 1965; 1969), it seemed reasonable to suppose that diphenylhydantoin exerts its action on this multi-enzyme system. A previous study by Rawson & Pincus (1968), clearly showed diphenylhydantoin 10^{-4}M to inhibit (Na^+,K^+)-activated ATPase by approximately 20% and to increase the inhibiting effects of ouabain. Many neuropharmacologically active drugs do not affect the (Na^+,K^+)-activated ATPase (Pincus & Giarman, 1967) and it is not clear how the diphenylhydantoin inhibition can be related to the anticonvulsant action of this drug.

The aim of the present work has been to determine the *in vivo* effect of diphenylhydantoin, given by mouth to rats over 20 weeks, on (Na^+,K^+)-activated ATPase. Experiments were also made to determine the effect of phenobarbitone. The results *in vivo* were compared with *in vitro* experiments, which involved a preliminary exposure of a synaptosomal membrane fraction to the drugs.

Much evidence has been accumulated to support the concept, that the (Na^+,K^+)-activated ATPase reaction proceeds through two main steps: a Mg^{2+}-dependent Na^+-activated transphosphorylation during which the terminal phosphate group of

ATP is bound to the protein moiety of the enzyme, and a Mg^{2+}-dependent K^+-activated dephosphorylation (Fahn, Koval & Albers, 1966). Furthermore, experiments in this laboratory have indicated that step 2) also hydrolyses the artificial substrates acetylphosphate and *p*-nitrophenylphosphate (Formby & Clausen, 1968; 1969) during a Mg^{2+}-dependent and K^+-activated reaction. Since the inhibition of (Na^+,K^+)-activated ATPase by ouabain seems to affect the K^+-activated entity, i.e. step 2), and ouabain and diphenylhydantoin inhibiting effects on the enzyme are supposed to be additive (Rawson & Pincus, 1968), two enzymic activities were determined: (Na^+,K^+)-activated ATPase with ATP as substrate, and K^+-activated phosphohydrolase with *p*-nitrophenylphosphate and acetylphosphate as substrates.

EXPERIMENTAL

Materials and methods

Chemicals of highest commercial purity from Sigma (USA) and Merck (W. Germany) were used. Diphenylhydantoin and phenobarbitone were purchased from DAK (Denmark).

Animals. Male Wistar rats weighing initially 175–200 g, were used. Since diphenylhydantoin was given in the drinking water (pH adjusted to 9), initial experiments were made to determine for each rat the amount of water drunk daily at a constant temperature of 25° and its optimal diet. This was calculated as 91·3 ± 8·4 ml per kg body weight per 24 h and on this basis the drugs were added to the drinking water to give each rat 3–4 mg diphenylhydantoin or 2–3 mg of phenobarbitone every 24 h.

Determination of diphenylhydantoin and phenobarbitone. The method of Huisman (1966) was used for determination of the drugs in serum.

Isolation of subcellular membrane fractions. The method of Whittaker (1966) was used with slight modifications. Whole rat brains removed immediately after decapittion, were homogenized in ice-cold 0·32 M sucrose. The homogenate was diluted to 10% of the original weight (w/v) and centrifuged at 900 *g* (10 min) at 0°. The supernatant was centrifuged 18,000 × *g* (60 min) at 0° and the sediment from this centrifugation was suspended in 0·32 M sucrose and layered on a sucrose gradient containing the layers: 1·2M and 0·8M. The final ratios were 1 : 1 : 1. After centrifugation at 50 000 *g* (145 min) at 0°, the fractions A (myelin), B (synaptosomes) and C (mitochondria) were isolated. The supernatant from the 18 000 *g* centrifugation was recentrifuged at 100 000 *g* (40 min) at 0° yielding the microsomal fraction M.

Determination of protein. The method of Lowry, Rosebrough & others (1951) was used with bovine serum albumin as standard.

Preincubation procedures for in vitro *measurements.* The synaptosomal membrane fraction B from the brains of control rats was exposed to various concentrations of either drug at 37° for 60 min. Aliquots of 100 ml (0·2 mg of protein) were then added to incubation media, to which the same amount of drug had been added, and the enzyme activity assayed.

Assay of enzyme activities. K^+-Activated phosphohydrolase with *p*-nitrophenylphosphate as substrate was assayed as previously described (Formby, 1968). Briefly, the reaction mixture contained 100 μl of aqueous suspension of one of the fractions A, B, C or M (0·2 mg protein) and 300 μl medium (5·0 mM *p*-nitrophenylphosphate as Tris-salt, 5·0 mM $MgCl_2$, 7·5 mM KCl and 50 mM Tris-HCl buffer pH 7·4). After 3 min incubation at 37°, the amount of *p*-nitrophenolate was determined spectrophoto-

metrically at 400 nm. The enzyme activity was expressed as mU (= nmol/min)/mg protein.

K^+-Activated phosphohydrolase with acetylphosphate as substrate was assayed as previously described (Formby, 1968). The reaction mixture contained 100 μl of aqueous suspension of one of the fractions A, B, C or M (0·2 mg protein) and 1000 μl medium (5·0 mM acetylphosphate as Tris-salt, 5·0 mM $MgCl_2$, 7·5 mM KCl and 100 mM Tris-HCl buffer pH 7·4). After 5 min incubation at 37°, the amount of acetylphosphate was determined by the acetohydroxamate method (Israel & Titus, 1967). The enzyme activity was expressed as mU (= nmol/min)/mg protein. (Na^+,K^+) ATPase reaction mixture contained 100 μl aqueous membrane suspension (0·2 mg protein) and 500 μl medium (5·0 mM ATP as Tris-salt, 5·0 mM $MgCl_2$, 100 mM NaCl 20 mM KCl and 50 mM Tris-HCl buffer pH 7·4). After 3 min incubation at 37°, the amount of orthophosphate was determined (Fiske & Subbarow, 1925). The enzyme activity was expressed as mU (= nmol/min)/mg protein.

Besides specific activity, a relative specific activity (RSA) was used and defined as the ratio of the percentage of recovered enzyme activity to the percentage of the recovered protein of each fraction.

Statistical evaluation. Each value of enzyme activity represents the mean of two individual determinations. From the values of each experimental and control group mean and standard error (s.e.) of the mean were calculated (n = 5), as well as the significance $P \leqslant 0{\cdot}025$ was used.

Results

The serum concentrations of diphenylhydantoin and phenobarbitone were respectively 109 ± 29 and 68 + 21 μg/ml of serum. Neither drug was detected in control groups.

Table 1. *The effect of long-term medication of diphenylhydantoin* (DPH) *and phenobarbitone* (PB) *to rats on* K^+*-activated phosphohydrolase and* $(Na,^+K^+)$*-activated ATPase in particulate membrane fractions of the rat brain. Specific activities expressed as* mU (= nmol/min)/mg *protein.*

Enzyme		Particulate subfraction A Specific activity	RSA	B Specific activity	RSA	C Specific activity	RSA	M Specific activity	RSA*
K^+-phosphohydrolase with *p*-nitrophenylphosphate as substrate	DPH	136 ± 14	1·1	152 ± 18	1·3	111 ± 9	1·0	135 ± 15	—
	PB	141 ± 11	1·0	110 ± 9	0·7	101 ±·8	0·7	120 ± 11	—
	Control	141 ± 13	1·0	155 ± 9	0·9	125 ± 11	0·9	123 ± 10	—
K^+ phosphohydrolase with acetylphosphate as substrate	DPH	382 ± 15	1·0	535 ± 28	1·6	456 ± 44	1·2	487 ± 29	—
	PB	359 ± 14	0·9	397 ± 14	1·0	228 ±·32	0·6	378 ± 28	—
	Control	385 ± 21	1·0	521 ± 17	1·3	499 ± 35	1·3	507 ± 24	—
(Na^+,K^+)ATPase	DPH	559 ± 29	1·1	1198 ± 32	2·4	835 ± 23	1·7	1304 ± 46	—
	PB	565 ± 48	1·1	1019 ± 34	2·0	644 ± 32	1·3	1158 ± 32	—
	Control	604 ± 48	1·1	1241 ± 39	2·3	899 ± 22	1·6	1356 ± 48	—

* Relative specific activity: the ratio of % recovered enzyme activity to % recovered protein of each fraction.

In vivo *effect of diphenylhydantoin.* Table 1 shows the results of the enzymic assays with particulate fractions isolated from the experimental and control groups. Concerning the specific activities, only non-significant ($P = 0{\cdot}025$) differences were calculated between the diphenylhydantoin treated rats and the control. Also, the relative specific activities (RSA) are of the same order, except K^+-activated phosphohydrolase with *p*-nitrophenylphosphate as substrate in fraction B, which is increased when compared to the control.

In vivo *effect of phenobarbitone* Table 1 shows that the specific activities of K^+-activated phosphohydrolase with acetylphosphate as substrate and (Na^+, K^+)-activated ATPase are significant decreased ($P = 0{\cdot}025$) in the particulate fractions B, C and M when compared to the controls; also decreased RSA-values were found.

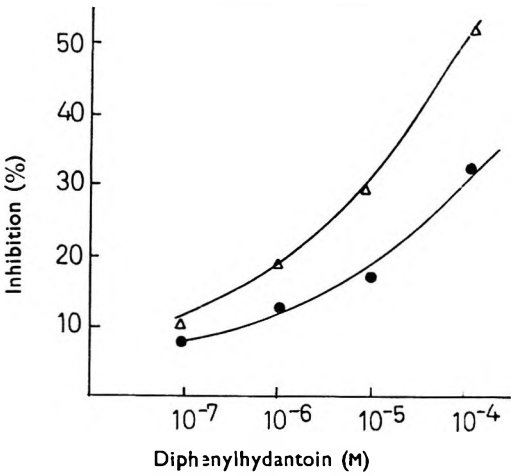

FIG. 1. The effect of pre-incubation with diphenylhydantoin on the specific activities of K^+-activated phosphohydrolase (with acetylphosphate as substrate) (△) and (Na^+,K^+)-activated ATPase (●). Synaptosomal membrane fraction B was preincubated at 37° for 1 h. The activity is expressed as % of the activity of the same enzyme preincubated for the same length of time, but in the absence of diphenylhydantoin.

In vitro *effect of diphenylhydantoin.* Fig. 1 shows the effect of various concentrations of diphenylhydantoin on the activities of K^+-activated phosphohydrolase (assayed with acetylphosphate as substrate) and (Na^+,K^+)-activated ATPase in the synaptosomal membrane fraction B. The synaptosomal membranes were pre-incubated at 37° for 60 min with the drug in various concentrations; membranes in pre-incubated for the same time but without the drug served as control. A half-maximal inhibition of K^+-activated phosphohydrolase was obtained with 10^{-4}M diphenylhydantoin. The same concentration inhibited (Na^+,K^+)-activated ATPase by about 30%. As already demonstrated by Rawson & Pincus (1968), ouabain acts as an additive inhibitor since a concentration of 10^{-5}M it increased the inhibition of K^+-activated phosphohydrolase by about 19% and that of (Na^+,K^+)-activated ATPase by about 26%.

In vitro *effect of phenobarbitone.* Corresponding experiments with phenobarbitone (10^{-4}M) indicated that it has no effect on K^+-activated phosphohydrolase and (Na^+, K^+)-activated ATPase.

DISCUSSION

Diphenylhydantoin, given orally for 20 weeks at 3–4 mg/24 h has no effect on K^+-activated phosphohydrolase or on (Na^+,K^+)-activated ATPase in any of the particulate fractions examined i.e. myelin fraction A, synaptosomal fraction B, mitochondrial fraction C and microsomal fraction M. However, in agreement with other findings (Rawson & Pincus, 1968), *in vitro* studies with this drug showed that pre-incubation of synaptosomal membranes at 37° for 1 h inhibited both K^+-activated phosphohydrolase and (Na^+,K^+)-activated ATPase. This inhibition was further increased by the

presence of ouabain, although the percentage increase was found to be significantly lower than if diphenylhydantoin was absent; this may indicate a competition between the two inhibitors (Conn 1965).

Although determination of diphenylhydantoin in brain tissue was not undertaken and possible effects of metabolites *in vivo* were ignored, it is obvious that the *in vivo* effect of diphenylhydantoin is different from that *in vitro*. One possibility is that the *in vitro* concentrations necessary to give inhibition are unphysiological and much higher than those existing *in vivo*. A second possibility is that there is a cation transport process, other than the (Na^+,K^+)-activated ATPase in neural tissue, upon which diphenylhydantoin acts. Such a "second pump" in nerves has been suggested by Rawson & Pincus (1968) and Pincus & Rawson (1969). A third possibility could be that diphenylhydantoin simply blocks the passive movement of sodium into the cell. Since diphenylhydantoin *in vivo* did not inhibit the (Na^+,K^+)-activated ATPase, the net result thus would be a decrease in intracellular sodium.

The *in vivo* effect of high doses of phenobarbitone is difficult to assess. Several lines of evidence suggest that phenobarbitone decreases the cerebral level of phosphorylated metabolites, leading to a reduced availability of ATP (Gey, Rutishauser & others, 1968; Kroner, Gutenberger & others, 1968). This suggests that phenobarbitone can interfere with the utilization of ATP in the brain, either directly or indirectly by depression of some energy-dependent processes like protein synthesis (Heald, 1960). Also this could explain the synergistic effect of phenobarbitone and diphenylhydantoin in the treatment of epilepsy, the former (for "petit-mal") indirectly inhibiting (Na^+, K^+)-activated ATPase and the latter (for "grand-mal") directly inhibiting the passive movement of sodium into the cell or a "second pump".

Finally, the difference in the *in vivo* and *in vitro* effects of these drugs must influence future design of experiments with anti-epileptic drugs.

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The inhibitory effect of non-steroidal anti-inflammatory agents on aggregation of red cells *in vitro*

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Aggregation of red cells of rat in saline suspension was induced by gelatin, high molecular weight dextran and fibrinogen. Except chloroquine, non-steroidal anti-inflammatory agents (NAIA) inhibited macromolecule-induced red cell aggregation *in vitro*. Glucocorticoids failed to inhibit red cell aggregation even in large concentrations. Of many drugs examined, only the NAIA and certain antihistamines, having experimental anti-inflammatory effects proved to be effective inhibitors of gelatin-induced red cell aggregation, *in vitro*.

Thrombosis in small vessels plays a significant role in the development of various inflammatory and necrotic reactions. The adhesive nature of platelets is a decisive factor in the development of a thrombus. *In vitro* aggregation of platelets induced by collagen particles is inhibited by certain non-steroidal anti-inflammatory agents (NAIA) (O'Brien, 1968; Görög & Kovács, 1968).

Though there can be no doubt that platelets are more prone to aggregate in response to various stimuli than other cells, most cells are liable to aggregate under appropriate conditions. The intra-arteriolar aggregation of erythrocytes (sludge phenomenon) is of pathophysiological significance. The red cell aggregates, transiently plug small vessels and can cause microcirculatory failure and thereby organ dysfunction. According to Bloch (1956) and Knisely (1963), intra-arteriolar aggregation of erythrocytes can never be demonstrated in the retinal vessels of healthy persons; when observable, it always indicates the presence of systemic disease.

In our studies, red cell aggregation induced by various macromolecules *in vitro* was significantly inhibited by NAIA. The investigation of many compounds revealed inhibition of gelatin-induced red cell aggregation to be highly specific to NAIA. Therefore, in addition to *in vivo* tests, this quick and specific *in vitro* method is recommended for use in the investigation of the anti-inflammatory effects of new compounds.

EXPERIMENTAL

Materials

Compounds were dissolved in 0·9% saline and the pH adjusted to 7·2–7·8 by addition of either NaOH or HCl. Aggregation of red cells was induced by gelatin (Knox Gelatin Inc., Johnstown, New York), by dextran (Koch-Light Labs., Ltd., Colnbrook, Bucks, England, M: 5–40 $\times$ 10^6) or by bovine fibrinogen (Phylaxia, Budapest, Hungary). The aggregating agents were dissolved in 0·9% saline. The

anti-inflammatory agents used were: salicylic acid, acetylsalicylic acid, phenylbutazone, sulphinpyrazone, mefenamic acid, flufenamic acid, cinchophen, indomethacin, chloroquine phosphate, benzydamine, ibufenac (4-isobutylphenylacetic acid), ibuprofen [2-(4-isobutylphenyl)propionic acid], BDH 7538 [4-(p-biphenylyl)-3-hydroxybutyric acid], ICI 54 450 [2-(4-chlorphenyl)thiazol-4-ylacetic acid, fenclozic acid], glyvenol (ethyl-3,5,6-tri-*o*-benzyl-D-glucofuranoside), prednisolone hemisuccinate sodium and dexamethasone 21-phosphate.

Methods

Adult rats of either sex were anaesthetized with pentobarbitone (45 mg/kg, intraperitoneally). Blood was allowed to flow from the abdominal aorta through a polyethylene cannula into a centrifuge tube containing heparin to give 20 U/ml final concentration. Of the blood collected from several animals in about 15–20 min, 3·0 ml samples were pipetted into Wassermann tubes. The blood was centrifuged for 20 min at 2000 rev/min, after which the plasma, leucocytes and platelets were removed and discarded. To the packed red cells, 2·0 ml of the solution prepared from the substance to be tested was added. In each series, two test and two control tubes were used. The tubes were sealed with rubber stoppers and the cells were cautiously suspended by ten inversions of the tube. After 5 min standing at 20°, 2·0 ml of 2% solutions of the aggregation-inducing agents were added to the tubes and the contents mixed by another ten inversions. The measure of aggregation was estimated by the erythrocyte sedimentation rate (ESR) which could be continually and accurately defined under the existing conditions. The tubes were held before an opal bulb to facilitate reading. Microscopic examination showed that increased ESR always implied enhanced cell-aggregation. 30 mm sedimentation was assigned a value of 100% for the control sample (no drugs). At the same time, sedimentation of test samples were determined, expressed as a percentage of the control and subtracted from 100% to give "Inhibition of aggregation (%)".

For *in vivo* experiments, male Wistar rats, 150–180 g, were treated subcutaneously with the substance to be tested and 1 h later the animals were exsanguinated under pentobarbitone anaesthesia. Samples of 3·5 ml of the heparinized blood were pipetted into Wassermann tubes; the aggregating solution (1·5 ml 2% gelatin) was added to each tube, the contents mixed and the ESR determined. The time for 30 mm sedimentation was measured and compared with the values obtained from saline-treated control rats. Two parallel readings were made for each animal.

RESULTS

Erythrocytes of the rat in heparinized whole blood or rat erythrocytes suspended in physiological saline show practically no sedimentation (ESR < 2 mm/h). On the addition of gelatin, swift aggregation of red cells is followed by rapid sedimentation (Coulson & Chalmers, 1964). Cellular agglomeration is looser on dextran or fibrinogen therefore the sedimentation is slower than on gelatin. As demonstrated in Fig. 1, the ESR is linear for the times between 5 and 35 mm values. Changes of temperature between 20–35° and of pH between 6·0–8·2 caused no alteration in the ESR. The sedimentation rate of erythrocyte suspensions can be determined accurately but not that of whole blood, therefore for the *in vitro* studies erythrocyte suspensions were used.

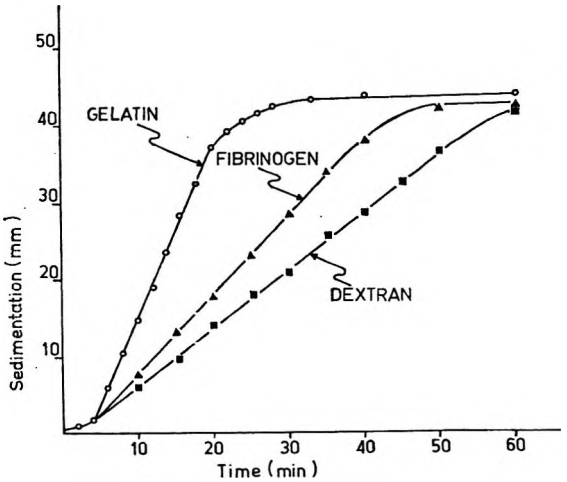

FIG. 1. Time-sedimentation curve of red cell aggregation induced by various macromolecules. Inhibitory effect of drugs (%) were calculated on the basis of 30 mm sedimentation of control sample for gelatin and fibrinogen and 20 mm sedimentation for dextran.

Table 1. *Effect of anti-inflammatory agents on red cell aggreggations induced by various macromolecules*

Drug (5×10^{-4}M)	Inhibition of aggregation (%)* induced by		
	Gelatin	Dextran	Fibrinogen
Salicylic acid	13·4	2·3	12·4
Acetylsalicylic acid	37·5	23·2	27·4
Phenylbutazone	100·0	89·4	76·3
Flufenamic acid	100·0	78·6	69·4
Mefenamic acid	96·0	68·2	57·7
Indomethacin	81·7	47·4	35·2
Cinchophen	89·2	39·7	37·1
Chloroquine	3·8	2·7	5·4
Benzydamine	63·4	23·4	18·8
Prednisolone	4·5	5·7	1·2
Dexamethasone	7·1	2·4	2·2

* = Calculated on the basis of readings when the ESR of control was 30 mm for gelatin and fibrinogen, and at control ESR = 20 mm for dextran.

Table 1 demonstrates that red cell aggregation induced by macromolecules is inhibited by NAIA. Of the three aggregating agents the effect of gelatin proved to be the most susceptible to inhibition with drugs, thus the gelatin-induced aggregation of red cell suspension was investigated further.

The effect of known anti-inflammatory agents is presented in Table 2. Aggregation was inhibited by all the compounds investigated except chloroquine and water-soluble glucocorticoids. The effect of the well-known compounds diminished in the order: phenylbutazone = flufenamic acid > mefenamic acid > indomethacin > ibufenac > acetylsalicylic acid > salicylic acid. Numerous compounds were investigated to decide whether the inhibition on gelatin-induced red cell aggregation is specific to NAIA or not. The effect of various compounds having different therapeutic use but all of them exerting a common antihistamine action—is presented in Table 3. The inhibitory potency of the effective compounds diminished in the order:

Table 2. *Effect of anti-inflammatory agents on gelatin-induced red cell aggregation* in vitro

Drug	Inhibition of aggregation (%)* at final concentration (M)							R†
	1×10^{-3}	5×10^{-4}	2×10^{-4}	1×10^{-4}	5×10^{-5}	1×10^{-5}	5×10^{-6}	
Salicylic acid‡	15·0	13·4						358·9
Acetylsalicylic acid	49·0	37·5	24·2					108·7
Phenylbutazone				95·0	89·4	51·7	26·2	1·0
Sulphinpyrazone			84·5	40·7	13·0			12·2
Mefenamic acid				89·7	55·4	27·3	8·6	4·6
Flufenamic acid					90·7	46·7	23·4	1·0
Cinchophen		89·2	30·8	14·5				32·6
Indomethacin		81·7	70·3	60·7	31·7	6·7		8·9
Chloroquine	2·4	3·8						—
Benzydamine	82·5	63·4	36·4	18·2				38·0
Ibufenac	91·0	65·0	44·8	17·5				28·2
Ibuprofen	90·0	64·8	37·9	15·0				30·4
BDH 7538		100·0	80·6	55·0	23·2			9·1
ICI 54 450	96·0	65·0	38·4	15·0				35·9
Glyvenol	68·7	40·3	20·2					73·9
Prednisolone	3·1	4·5		2·2				—
Dexamethasone	4·3	7·1		0·5				—

* = Inhibition < 15% is significant ($P > 0{\cdot}001$).
† = Relative potency (phenylbutazone = 1·0), calculated on the basis of 50% inhibitory concentrations.
‡ = Salicylic acid produced 50% inhibition in $3{\cdot}3 \times 10^{-3}$M concentration.

Table 3. *Effect of antihistamines on gelatin-induced red cell aggregation* in vitro

Drug	Inhibition of aggregation (%)* at final concentration (M)			Antagonism of i.v. histamine† (oral ED50 mg/kg)
	5×10^{-4}	2×10^{-4}	1×10^{-4}	
Diphenhydramine	4·8			1·02 ± 0·5
Chloropyramine	5·0			
Diethazin	4·5			
Tripelenamine	2·4			0·50 ± 0·08
Amitryptiline	10·4			0·21 ± 0·03
Promethazine	18·0	4·1		
Imipramine	42·7	27·3	4·7	7·43 ± 1·60
Desipramine	30·0	17·4	2·2	
Chlorpromazine	49·2	25·0	10·2	4·10 ± 2·80
Cyproheptadine	39·2	18·2	2·2	0·08 ± 0·02

* = Inhibition > 15% is significant ($P < 0{\cdot}001$).
† = All data are obtained from the paper of Lish, Robbins & Peters (1966).

chlorpromazine > imipramine > cyproheptadine > promethazine. The aggregation-inhibitory effects of antihistamines is much weaker than that exhibited by the NAIA. The inhibitory effect on aggregation is independent of antihistamine potency. It is noteworthy that experimentally induced inflammations have been inhibited by chlorpromazine and promethazine (Lish, Alberts & others, 1960; Brown & Robson, 1964), by imipramine (Tangri, Saxena & others, 1966) and by cyproheptadine (Selye & Somogyi, 1967), while evidence that the experimental anti-inflammatory effects of antihistamines do not influence red cell aggregation is not available. Some 100 drugs in widely differing categories were found inactive.

The efficacy of several NAIA *in vivo* is illustrated in Fig. 2. Gelatin-induced red cell aggregation was significantly inhibited by pretreatment of the rats with non-toxic doses of phenylbutazone or acetylsalicylic acid, while flufenamic acid or indomethacin have inhibitory effects only in toxic doses. Unlike acetylsalicylic acid, sodium salicylate was ineffectual *in vivo*.

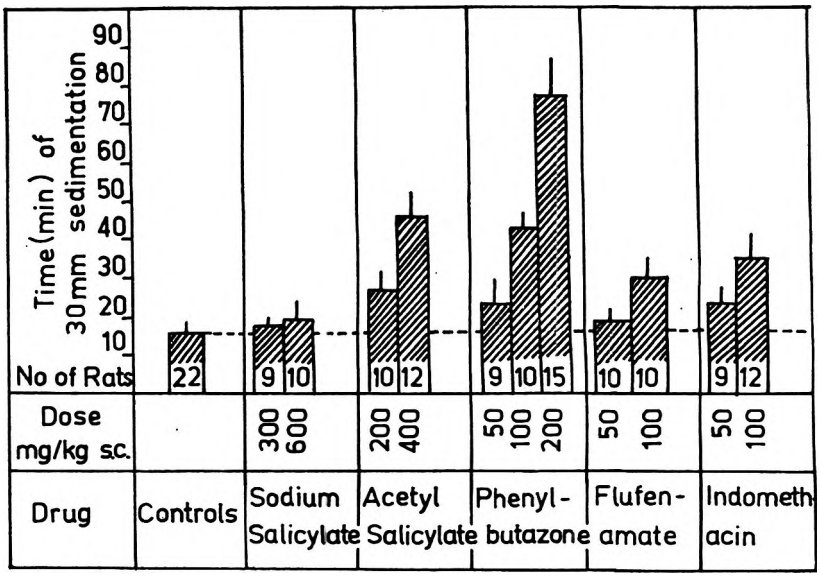

FIG. 2. *In vivo* effect of anti-inflammatory drugs on gelatin-induced red cell aggregation. Vertical lines show the standard errors. According to a separate experiment, the drugs in the applied doses caused no death within 6 h. After 48 h, flufenamate, 100 mg/kg dose, was lethal to 3 of 10 rats, indomethacin in 50 and 100 mg/kg doses was lethal to 9 and 10 of 10 rats, respectively.

DISCUSSION

The NAIA cause several *in vitro* effects which can be used to investigate the mechanism of action of new anti-inflammatory agents. NAIA can inhibit heat-induced denaturation of proteins (Mizushima & Kobayashi, 1968), the heat-induced haemolysis of erythrocytes (Brown, Mackey & Riggilo, 1967), the biosynthesis of mucopolysaccharides in the connective tissue (Whitehouse & Boström, 1962). In these tests numerous compounds have been effective which have not shown any anti-inflammatory action either under experimental conditions or in the therapy.

As in all the above tests, as well as in our method, the effect of drugs was observed in plasma protein-free medium, it was justified to compare the effective concentrations. Of the *in vitro* methods listed, that based on the inhibitory effect of NAIA on gelatin-induced red cell aggregation proved to be the most sensitive to the influence of NAIA.

Despite the essential differences in sensitivity and selectivity of these *in vitro* methods, the order of diminishing potency proved by every test to be: flufenamic acid $\geqq$ phenylbutazone $>$ indomethacin $>$ ibufenac $>$ acetylsalicylic acid $>$ salicylic acid. Of the anti-inflammatory agents investigated, glucocorticoids, even in large concentrations, failed to inhibit red cell aggregation. This is an essential difference from the method based on inhibition of heat-induced haemolysis of erythrocytes in which various steroids are active (Brown & Mackey, 1968). Of the NAIA, chloroquine alone failed to inhibit red cell aggregation. This is unexpected as it is the only drug to be recommended as a "desludging" agent on the basis of its therapeutic action (Sandler, Ilaki & Lawson, 1963). On the other hand, chloroquine is the only NAIA the efficacy of which has not been fully confirmed by acute experiments.

According to Knisely, Bloch & others (1950) sludging is probably unrelated to the rouleaux formation associated with the ESR, the increase in which is thought to be due to a much looser aggregation of red cells. This may be the cause of differences between our results based on erythrocyte aggregation and the results of Ruhenstroth-Bauer, Brittinger & others (1960) relating to the inhibition of increased ESR. The latter authors concluded that the increased ESR is caused by specific plasma proteins called agglomerines and their effect can be inhibited partially or completely. According to the authors, complete blockers include salicylic acid, gentisic acid, phenylbutazone, and cinchophen, while partial blockers are cortisone, antazoline, and chloroquine. These findings show the wide differences existing between the inhibition of increased ESR and the macromolecule-induced red cell aggregation.

However, the susceptibility of red cell aggregation to inhibition and the inhibition of collagen-induced aggregation of platelets, *in vitro* have some similarities. Glucocorticoids have proved to be ineffective in both tests and the order of effectiveness of NAIA is also very similar. The only exception is chloroquine which, according to our experiments, inhibits the collagen-induced platelet aggregation but fails in the gelatin-induced red cell aggregation.

For the most effective NAIA, the inhibitory concentrations on the aggregation of red cells *in vitro* were those usually attained in patients after oral medication. Nevertheless only phenylbutazone and acetylsalicylic acid were effective in the *in vivo* experiments. Flufenamic acid, the most effective compound *in vitro* had the minimal *in vivo* effect. This discrepancy resembles the uncoupling of oxidative phosphorylation by flufenamic acid *in vitro* and *in vivo* (Whitehouse, 1965). According to Whitehouse, the ineffectiveness of anthranilates in biochemical tests *in vivo* is due to the excessively lipophil nature of these compounds barring the development of effective blood levels.

On the evidence we have derived about the mechanism responsible for the inhibitory effect of NAIA on red cell aggregation, the site of action is most probably on actomyosin-like contractile protein having ATPase activity and situated on the outer surface of the erythrocyte. This contractile protein plays an important role in maintaining the form of the erythrocyte and the distribution of the surface charge on the outer membrane. Effective NAIA bind to this contractile protein and inhibit its ATPase and contractile property. The results of our biochemical findings which have furnished the basis of the above hypothesis is to be published later. Presumably, adenosinetriphosphate (ATP) utilization is inhibited by NAIA through a similar mechanism not only in the red cell membrane (and most probably in the membrane of platelets as well), but also in the true target of these drugs: in the connective tissue. At all events, the highly selective inhibition of red cell aggregation by NAIA *in vitro* suggests the existence of a relation between the effect in the connective tissues and that exerted on the erythrocyte membranes.

With most *in vitro* biochemical tests (including that in the present paper) the order of efficacy of the effective compounds differs from that registered in *in vivo* tests for anti-inflammatory action (carrageenan-induced oedema test). For this reason Glenn (1969) disclaims any relation between various *in vitro* biochemical effects and anti-inflammatory action, and regards the former as manifestations of other side-effects of NAIA, for instance those exerted on coagulations.

We think that every new *in vitro* effect of NAIA takes us nearer to understanding the mode of action of these compounds. At the same time we emphasize the

importance of elucidating other factors (drug-metabolism, lipid-solubility, binding to plasma proteins, pK_a) that are responsible for any discrepancy between *in vitro* biochemical effects and *in vivo* anti-inflammatory potency.

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Prevention of the reserpine effect on rat salivary gland noradrenaline by inhibitors of monoamine oxidase and catechol-*O*-methyl transferase

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The roles of monoamine oxidase and catechol-*O*-methyl transferase for the inactivation of noradrenaline after reserpine treatment have been investigated in rat salivary glands *in vivo*. Inhibition of monoamine oxidase by nialamide retarded the reserpine-induced disappearance of noradrenaline in the glands, whereas inhibition of catechol-*O*-methyl transferase by 4-tropolone acetamide did not. However, when both enzymes were inhibited, reserpine produced a significantly slower disappearance rate of noradrenaline compared with inhibition of monoamine oxidase alone. Thus, after reserpine, most of the noradrenaline is metabolized by monoamine oxidase. When this enzyme is inhibited catabolism by catechol-*O*-methyl transferase appears to play a part in the reduction of noradrenaline induced by reserpine.

Reserpine is known to deplete the tissue stores of catecholamines (Carlsson, Rosengren & others, 1957; Burn & Rand, 1958), and this is thought to be due to it blocking the uptake of the amines into storage granules. Reserpine has also been reported to alter catecholamine metabolism. Thus, there is an increased catabolism of catecholamines by monoamine oxidase (MAO) after reserpine treatment (for review see Carlsson, 1965). Evidence has been presented that in rat salivary glands this increased deamination occurs intraneuronally (Jonason, 1969a). In the present experiments the relative roles of MAO and catechol-*O*-methyl transferase (COMT) for the inactivation of noradrenaline in rat salivary glands after reserpine have been investigated. This has been done by measuring the reserpine-induced disappearance of noradrenaline after inhibition of MAO or COMT, or both.

EXPERIMENTAL

Adult male Sprague-Dawley rats, about 200 g, were treated with reserpine, 1 mg/kg, intraperitoneally. Some of the animals were pretreated with the MAO inhibitor nialamide (100 mg/kg, i.p.) 2 h before, or the COMT inhibitor 4-tropolone acetamide (100 mg/kg, i.p.) 30 min before the reserpine administration. Another group of animals received a combination of these two enzyme inhibitors as pretreatment. The rats were killed by a blow on the head at different times after the reserpine administration. The submaxillary plus the sublingual glands on each side were removed as soon as possible, weighed and homogenized in 10 ml 0·4N perchloric acid by an "Ultra-Turrax" homogenizer. The noradrenaline was determined spectrophotofluorometrically after cation exchange chromatography (Bertler, Carlsson & Rosengren, 1958).

Table 1. *Effect of inhibition of monoamine oxidase or catechol-O-methyl transferase, or both, on the reserpine-induced noradrenaline disappearance rate in rat salivary glands.* Rats were treated with reserpine (1 mg/kg, i.p.) and the noradrenaline content of the submaxillary plus the sublingual glands was determined at different time intervals after the injection. Some of the animals were pretreated with the monoamine oxidase inhibitor nialamide (100 mg/kg, i.p.) 2 h before the reserpine administration or the catechol-*O*-methyl transferase inhibitor 4-tropolone acetamide (100 mg/kg, i.p.) 30 min before the reserpine administration. Another group of the animals received a combination of the two drugs as pretreatment.

s.e. represents the standard error of the mean and n represents the number of experiments.

		Noradrenaline content μg/g tissue after reserpine treatment for:			
		0 h	4 h	6 h	8 h
Reserpine	Mean	1·183	0·014	0·007	0·006
	s.e.	0·068	0·0071	0·0042	0·0043
	n	8	5	4	10
Nialamide + reserpine	Mean	—	0·235	0·122	0·108
	s.e.	—	0·021	0·0114	0·034
	n	—	5	6	10
4-Tropolone acetamide + reserpine	Mean	—	0·015	—	0·001
	s.e.	—	0·0057	—	0·00047
	n	—	6	—	10
Nialamide + 4-tropolone acetamide + reserpine	Mean	—	0·383	0·236	0·103
	s.e.	—	0·045	0·045	0·034
	n	—	6	6	10

RESULTS

The results are presented in Table 1. Four h after the reserpine administration the noradrenaline content of the salivary glands was reduced to a very low concentration which was maintained for the rest of the investigated time intervals. Inhibition of COMT by 4-tropolone acetamide did not affect this reserpine-induced noradrenaline reduction. Pretreatment of the rats with nialamide (100 mg/kg, i.p.) does not significantly increase the noradrenaline content in the salivary glands within 2 h (Jonason, 1969a). However, after inhibition of MAO by nialamide, reserpine produced a much slower disappearance rate of the salivary gland noradrenaline, resulting in significantly higher noradrenaline content in the MAO-inhibited glands than after reserpine alone at all time intervals investigated ($P < 0{\cdot}001$).

Pretreatment of the animals with both the MAO inhibitor nialamide and the COMT inhibitor 4-tropolone acetamide resulted in a further reduction of the reserpine-induced noradrenaline disappearance rate. The noradrenaline content of the salivary glands was found to be significantly higher in the nialamide plus 4-tropolone acetamide-treated glands than in the nialamide-treated glands 4 and 6 h after reserpine ($P < 0{\cdot}005$ and $P < 0{\cdot}025$, respectively). Eight h after reserpine treatment there was no difference between the two pretreatments.

DISCUSSION

Kalsner & Nickerson (1969) recently reported that interference with intraneuronal storage by reserpine results in active amine being inactivated by COMT rather than by MAO in rabbit aortic strips. However, these results were obtained after adding noradrenaline exogenously to muscle baths. From the above experiments, dealing with endogenous noradrenaline levels after reserpine, it is obvious that MAO inhibition significantly reduced the rate of noradrenaline decrease after reserpine whereas inhibition of COMT did not affect the reserpine-induced noradrenaline reduction. The prevention of the reserpine effect on the noradrenaline by a MAO inhibitor but not by a COMT inhibitor supports the view that after reserpine treatment most of the noradrenaline is metabolized by means of MAO (Carlsson & others, 1957; Carlsson & Hillarp, 1962; Kopin, 1964; Malmfors, 1965). This oxidative deamination after reserpine treatment has been shown to occur intraneuronally (Jonason, 1969a).

However, the role of COMT has also been partially revealed in the present investigation. If both MAO and COMT were inhibited, the prevention of the reserpine effect on noradrenaline was augmented. Thus, after MAO inhibition followed by reserpine treatment, the noradrenaline is to a certain extent 3-*O*-methylated by COMT. This enzyme has been shown to be localized to the parenchymal cells in rat salivary glands (Jonason, 1969b,c). Jonason (1969a) has demonstrated that after treatment with nialamide the reserpine-induced noradrenaline reduction is slower in atrophied rat salivary glands compared with intact ones. Since there is a severe loss of COMT activity in atrophied glands (Jonason, 1969b,c), the reason for this difference may well be the lack of COMT. The data from the present investigation support this interpretation. Thus, it seems probable that after MAO inhibition followed by reserpine treatment the noradrenaline leaves the adrenergic nerves and is 3-*O*-methylated by means of parenchymal COMT or is transported unchanged from the neuroeffector units by the blood.

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The interaction of hemicholinium-3 and oxotremorine in isolated organ preparations

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On isolated ileum preparations of the rat and guinea-pig, hemicholinium-3 antagonizes contractions elicited by acetylcholine and oxotremorine to the same extent. Hemicholinium-3 was a mild antagonist to acetylcholine but a stronger one to oxotremorine and carbachol on the ileum of the rabbit. Whereas hemicholinium-3 has no anti-acetylcholine activity on the isolated urinary bladder of the rat, it antagonizes the contractions elicited by oxotremorine and carbachol, and acetylcholine after eserine. Morphine has an anti-oxotremorine activity on this organ. Increasing concentrations of oxotremorine release increasing amounts of acetylcholine from the rat isolated intestine.

The peripheral cholinergic action of oxotremorine is generally thought to be of direct postsynaptic origin because cholinesterase-blocking agents do not aggravate the fall in blood pressure it produces (Cho, Haslett & Jenden, 1962; Haslett, 1963) and because its spasmogenic effect on the guinea-pig intestine cannot be reduced with morphine, which is known to inhibit the release of acetylcholine (Lévy & Michel-Ber, 1967a). Lévy & Michel-Ber have also shown that eserine potentiates the action of oxotremorine on striated muscle preparations like the leech dorsal muscle and rat diaphragm. Paton & Aboo Zar (1968) report a possible action of tremorine on the nerve plexus.

Doubt has lately been cast on the direct cholinergic action of oxotremorine by the finding that the compound raises the level of acetylcholine in the brain of the rat (Holmstedt, Lundgren & others, 1965; Holmstedt, 1967) and the mouse (Lévy & Michel-Ber, 1967b). The tremor response to tremorine is depressed by compounds that inhibit acetylcholine synthesis, like hemicholinium-3 in chicks (Bowman & Osuide, 1968) and triethylcholine in rats (Slater & Rogers, 1968). This would appear to argue in favour of an indirect mechanism of action in the central nervous system both for oxotremorine and tremorine.

The present experiments were designed to find if the peripheral effects of oxotremorine could be influenced with hemicholinium-3. For this, a preparation was required in which the atropine-like activity of hemicholinium-3, observed by Bieger Lüllmann & Wassermann (1968) in the isolated atrium of the guinea-pig, would not be present. The urinary bladder of the rat proved satisfactory.

EXPERIMENTAL

The ileum from rats, guinea-pigs and rabbits, and the urinary bladder from rats were used. Contractions were recorded kymographically using an isotonic lever. The temperature of the oxygenated solution was maintained at 37° for the ileum and at 34° for the bladder. The composition of the solution used for all preparations was:

NaCl, 7·5; KCl, 0·41; $CaCl_2$, 0·24; $NaHCO_3$, 0·24; NaH_2PO_4, 0·14; glucose 1·0 g and distilled water 1000 ml. Contact time for the spasmogenic agents (acetylcholine oxotremorine and carbachol) was 0·5 min with ileum preparations and 1–2 min with bladders; the agents were always applied 15 min after hemicholinium-3. In analysing cumulative dose-effect relations on the rat isolated bladder the end-concentrations of the spasmogenic compounds were usually doubled at 1·5 min intervals.

To examine the acetylcholine-releasing action of oxotremorine, the small intestine, without the duodenum, of rats fasted for 24 h was cut into 9 pieces of roughly equal length, of which the 1st, 4th and 7th piece, the 2nd, 5th, and 8th piece, and the 3rd, 6th, and 9th piece were placed in separate vessels; in this manner each vessel contained 1·5–2·0 g of gut. In 8 ml of a solution containing eserine 10 μg/ml and gassed with O_2 at 37°, the pieces of gut were incubated for 10 min in the presence or absence of oxtremorine 5, 10 or 20 μg/ml. After 1 : 1·15 dilution with distilled water, the amount of acetylcholine in the incubation medium was determined on frog rectus abdominis muscle suspended in Ringer solution containing eserine (10 μg/ml). To identify acetylcholine as the agent producing the contractions the extracts were boiled in an alkaline medium or the agent was antagonized by tubocurarine. Oxotremorine itself produces no contractions even at concentrations as high as 20–30 μg/ml.

RESULTS

Rat isolated ileum. Hemicholinium-3, when used in doses of 30–300 μg/ml, antagonized acetylcholine- and oxotremorine-elicited contractions to the same extent.

Guinea-pig isolated ileum. Results similar to those with rat ileum were obtained: hemicholinium-3 (100 μg/ml) prevented the action of both acetylcholine and oxotremorine to approximately the same extent.

Rat isolated urinary bladder. Hemicholinium-3 (30 μg/ml given 1 to 3 times) usually did not affect acetylcholine-induced contractions but sometimes increased them. In contrast, hemicholinium-3 reduced the effects of oxotremorine and carbachol by 60 to 70% (Fig. 1). At higher concentrations, 60–200 μg/ml, hemicholinium-3 antagonized acetylcholine contractions 10–20%, whereas those caused by oxotremorine or carbachol were reduced 60–100%.

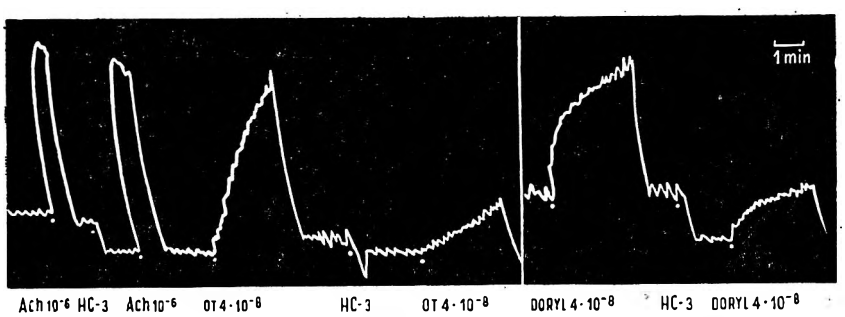

FIG. 1. Contractions of a rat urinary bladder, g/ml concentrations. HC-3: 3×10^{-5} g/ml hemicholinium-3 (Doryl = carbachol). OT = oxotremorine. Hemicholinium-3 reduces the effects of oxotremorine and carbachol by 60–70%.

In the presence of eserine (1 μg/ml), low concentrations of hemicholinium-3 (30 μg/ml) inhibited the action of acetylcholine to the same extent as it did that of the other two spasmogenic substances (Fig. 2).

FIG. 2. Contractions of a rat urinary bladder in the presence of 10^{-6} g/ml eserine. A low concentration of hemicholinium-3 now inhibits acetylcholine action to the same extent as the other spasmogens. Concentrations in g/ml.

At 10 μg/ml hemicholinium-3 was inactive, whilst at 30 μg/ml it produced a shift to the right of the dose-response curve for oxotremorine but the maximum remained unaffected (Fig. 3), a similar shift was observed with carbachol, and also with acetylcholine, but eserine was then required. The antagonistic effects against each of the three compounds was nearly identical: the pA_2 mean values of 4 experiments was 4·59 $\pm$ 0·41 for oxotremorine, 4·58 $\pm$ 0·29 for carbachol, and 4·695 $\pm$ 0·31 for acetylcholine ($\pm$ values: probability intervals, P 95).

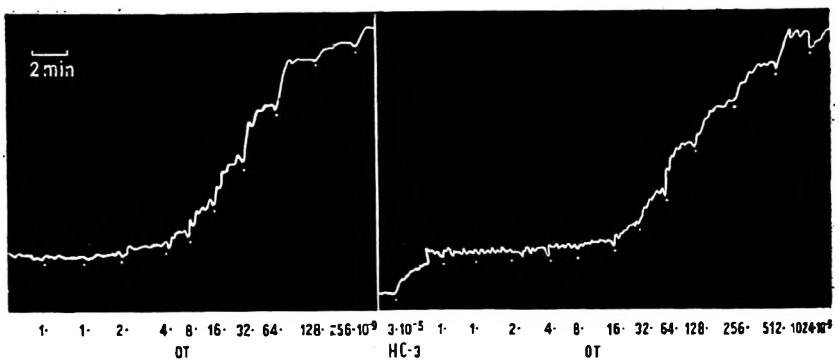

FIG. 3. Registrogram of cumulative dose-response curves obtained with oxotremorine (OT, n $\times$ 10^{-9} g/ml) on the isolated urinary bladder of the rat before and after hemicholinium-3 (HC-3, 3 $\times$ 10^{-5} g/ml).

In ten experiments with the bladder it was found invariably that morphine (50 μg/ml) increased the magnitude of acetylcholine-induced contractions by about 50% but reduced oxotremorine-elicited contractions by some 25%. Eserine 0·1 μg/ml increased the effect of low oxotremorine concentrations 1·5–2·5 fold.

Rabbit isolated ileum. At 30 μg/ml, hemicholinium-3 had no effect on acetylcholine-induced contractions but it reduced by about 30% those elicited by oxotremorine. At 100–300 μg/ml, hemicholinium-3 was a mild antagonist to acetylcholine (up to 23%) and a strong antagonist to oxotremorine and carbachol (40 to 80%) (Fig. 4).

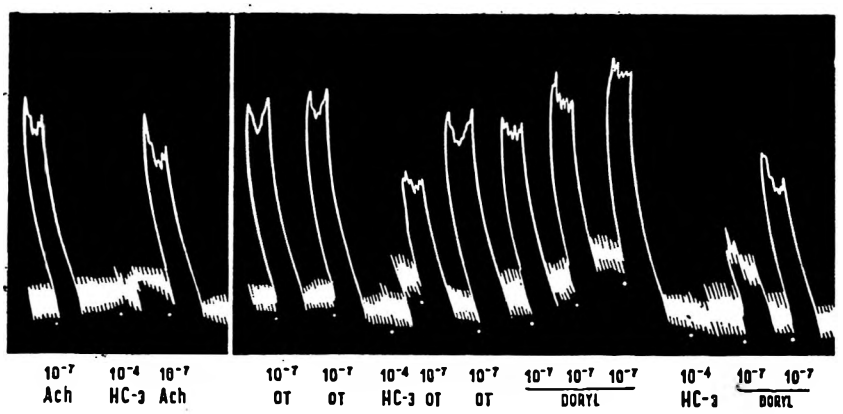

FIG. 4. Contractions of a rabbit ileum, g/ml concentrations (Doryl = carbachol). At 3×10^{-5} g/ml, hemicholinium-3 had no effect on acetylcholine-induced contractions but it reduced by about 30% those elicited by oxotremorine. At 10^{-4} g/ml, hemicholinium-3 was a mild antagonist to acetylcholine (up to 23%) and a strong antagonist to oxotremorine and carbachol (40 to 80%).

Acetylcholine release from rat gut. In only two of our experiments was acetylcholine released in appreciable amounts from rat isolated gut incubated in the absence of oxotremorine (Table 1). In the presence of oxotremorine, however, there was release, the rate of which was concentration dependent, doubling the oxotremorine concentration increasing the rate 1·3 to 3 fold. Boiling in alkaline medium or application of tubocurarine arrested activity in each case.

Table 1. *The effect of oxotremorine on the release of acetycholine from the isolated rat intestine, in ug/g of tissue.*

	Oxotremorine (μg/ml)			
	0	5	10	20
Acetylcholine μg/g	0·004–0·04	0·008–0·15	0·003–0·33	0·005–0·37
x/n	2/14	3/4	12/14	8/8

n = number of determinations x = number giving measurable release

DISCUSSION

Because of the atropine-like effect which hemicholinium-3 exerts on the small intestine of the rat and the guinea-pig, these preparations cannot be used to examine the "indirect cholinergic" action of oxotremorine. As the atropine-like activity of hemicholinium-3 is weak on the rabbit intestine, this preparation is more suitable for this purpose. But the rat isolated bladder is most appropriate because hemicholinium-3 has no anti-acetylcholine activity on it. It also has no parasympathetic ganglia, so is unaffected by ganglion stimulants (Hukovič, Rand & Vanov, 1965).

Our results with rat bladder preparations and our incubation experiments have provided evidence that both oxotremorine and carbachol possess properties that can be inhibited peripherally by hemicholinium-3, i.e. the release or activation of acetylcholine. The indirect, acetylcholine-releasing action of carbachol has been described by McKinstry & Koelle (1967) in sympathetic ganglia.

But hemicholinium-3 inhibits acetylcholine only in the presence of eserine, while markedly depressing carbachol-elicited contractions in its absence. We incline to the view that the immediate and short-acting action of acetylcholine destroyed by cholinesterase is a postsynaptic action whereas the lasting action of carbachol or that of acetylcholine after a cholinesterase inhibitor mobilizes or activates presynaptic acetylcholine—a view based on an earlier assumption of Koelle (1961; 1962).

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Specific blockade of spasmogens by β-receptor stimulation with nylidrin and isoprenaline

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Nylidrin, a β-adrenoreceptor stimulant drug, blocks the spasmogenic action of acetylcholine, histamine and barium on guinea-pig ileum *in vitro* and bronchial muscles *in vivo*. This action is antagonized by the β-receptor blocking agent, propranolol. It is suggested that the antispasmodic effect of nylidrin is mediated through the activation of β-receptors. Nylidrin seems to be less potent but longer acting in its antispasmodic action than isoprenaline.

Nylidrin is a sympathomimetic agent with a β-adrenoreceptor stimulant action (Goodman & Gilman, 1965); it is used clinically for peripheral vascular disorders (Freedman, 1955; Caliva, Eich & others, 1959).

We have investigated whether nylidrin has an inhibitory action against spasm produced by spasmogens acting through receptors or directly on smooth muscle. We have also sought to explain any inhibition and have compared the effects of the drug with those of isoprenaline.

EXPERIMENTAL

Effect on spasm produced by spasmogens in guinea-pig ileum. Guinea-pigs, 300–400 g, were killed by a blow on the head and bled. A terminal section of ileum was removed, cleaned and set up in a 25 ml bath containing aerated Tyrode solution at 32–34°. Contractions were recorded with a frontal writing lever. The effect of nylidrin ($2{\cdot}4 \times 10^{-8}$ and 8×10^{-7} g/ml) with 1 min contact was studied on spasm produced by histamine, acetylcholine and barium. In other experiments, the effects of nylidrin was studied after blockade of β-adrenoreceptors by propranolol. The inhibitory action of nylidrin against histamine was compared with that of similar doses of isoprenaline.

Effect of bronchoconstriction produced by a histamine aerosol in guinea-pigs. Male guinea-pigs, 400 g, in groups of six, were exposed to a histamine aerosol of 20 mg/ml. When an animal collapsed (dropping of the neck), it was revived by artificial respiration. The time from starting the aerosol to the collapse of the animal was noted. One group acted as control, another group received nylidrin, 5 mg/kg intraperitonally, 15 min before exposure to the aerosol. A third group was treated with propranolol followed at 15 min by the histamine. A fourth group received propranolol followed after 20 min by nylidrin and then 15 min later by the histamine. Cross over tests were made.

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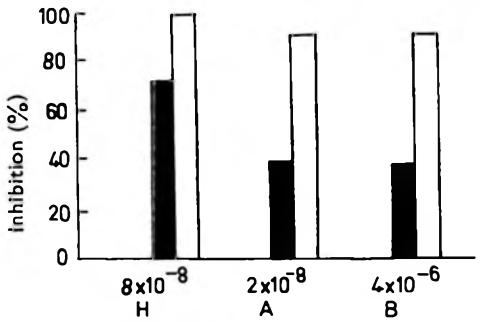

FIG. 1. Inhibitory action of nylidrin against histamine (H), acetylcholine (A) and barium (B). Solid columns represent nylidrin $2{\cdot}4 \times 10^{-8}$ g/ml and open columns nylidrin 8×10^{-7} g/ml.

Drugs used

Nylidrin hydrochloride, histamine acid phosphate, acetylcholine bromide, barium chloride, propranolol hydrochloride, isoprenaline sulphate. Concentrations expressed are of the salts, and are the final concentrations (g/ml) in the bath.

RESULTS

Effect of nylidrin on acetylcholine-, histamine- and barium-induced spasm on guinea-pig ileum. Fig. 1 shows the inhibitory action of nylidrin on the height of contraction produced by histamine (8×10^{-8}), acetylcholine (2×10^{-8}) and barium 4×10^{-6}). Nylidrin was added to the bath followed 1 min later by histamine, acetylcholine or barium. Nylidrin ($2{\cdot}4 \times 10^{-8}$) reduced the contraction of histamine on average by 71% ($n = 9$) and of acetylcholine on average by 36%. Recovery of the acetylcholine contraction took only 5 min, while for histamine it was incomplete after 60 min. Thus the inhibitory action of nylidrin was more potent and also longer lasting against histamine than acetylcholine.

At a higher concentration (8×10^{-7}) nylidrin completely inhibited the responses to acetylcholine and histamine. Recovery to acetylcholine was complete in 30 min, but for histamine it was not complete 90 min later.

An examination of comparative effect of nylidrin ($2{\cdot}4 \times 10^{-8}$) and isoprenaline ($2{\cdot}4 \times 10^{-8}$) on spasm produced by histamine (8×10^{-8}) showed isoprenaline to be the more potent at equal dosage. It completely blocked the contraction of histamine, while nylidrin reduced the contraction of histamine by 60% ($n = 4$) on the ileum of one animal. However, recovery of histamine contraction after isoprenaline took only 12 min, and after nylidrin it was 35 min. This showed that although isoprenaline was more potent than nylidrin in identical doses in antagonizing histamine spasm, its effects were shorter lasting.

Effect of β-adrenoreceptor blockade by propranolol. β-Adrenoreceptors were blocked by propranolol (2×10^{-7} mg/ml), a dose having no effect on histamine and acetylcholine. After propranolol, nylidrin ($2{\cdot}4 \times 10^{-8}$ mg/ml) was unable to inhibit the spasm induced by histamine or acetylcholine.

Effect on bronchoconstriction induced by histamine aerosol in guinea-pig. In all control animals, the histamine aerosol (20 ml) produced bronchospasm (as seen by collapse) within 3 min. Nylidrin (5 mg/kg) alone extended the collapse time to 8 min. Propranolol (10 mg/kg) reduced the collapse time to 2 min and caused 50% mortality. Propranolol followed by nylidrin gave a near collapse time of 2·5 min and a 33% (2/6) mortality.

Thus, nylidrin (5 mg/kg) gave significantly protection against histamine induced bronchospasm, which was completely antagonized by propranolol.

DISCUSSION

The results suggest that nylidrin possesses an inhibitory action against histamine-, acetylcholine- and barium-induced spasm of guinea-pig ileum. Further, nylidrin possesses a greater inhibitory action against histamine than against acetylcholine. These results are similar to those obtained for other catecholamines such as isoprenaline, noradrenaline and adrenaline (Wilson, 1964).

The comparative study of the inhibitory action of nylidrin and isoprenaline in similar doses ($2{\cdot}4 \times 10^{-8}$) suggest that isoprenaline is more potent than nylidrin against histamine-induced spasm, but the effects of nylidrin seem to be the longer lasting.

When β-adrenoreceptors are blocked by propranolol, nylidrin fails to antagonize the spasmogenic action of histamine and acetylcholine on ileum, thus suggesting the involvement of β-adrenoreceptors in the inhibitory action of nylidrin. Similar observations have been made by Farmer & Lehrer (1966) with isoprenaline. These authors have also demonstrated that in isolated human myometrium, which contains few or no β-receptors, isoprenaline does not antagonize the action of histamine and acetylcholine.

These observations support the view that the inhibitory action of nylidrin on the spasmogenic drugs is through the activation of β-adrenoreceptors.

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Cutaneous vascular permeability factors (histamine, 5-hydroxytryptamine, bradykinin) and passive cutaneous anaphylaxis in sheep

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The cutaneous blood vessels of sheep are more sensitive to histamine than those of laboratory rodents. The threshold dose in sheep was 0·0025 μg histamine. The ovine vessels are 70 to 100 times less sensitive to 5-hydroxytryptamine (5-HT) than to histamine, but only four times less sensitive to bradykinin than to histamine. The effects of compound 48/80 are antagonized both by the antihistamine agent mepyramine and by the anti-5-HT agent methysergide, which suggests that compound 48/80 may release 5-HT in addition to histamine in sheep. The capillary-damaging effects of passive cutaneous anaphylaxis in sheep are antagonized by methysergide and by sodium meclofenamate—an agent which antagonizes kinins and slow-reacting substance. The antihistamine agent mepyramine has a small anti-anaphylactic effect, whereas promethazine—a less specific antihistamine—offers more protection to the blood vessels against local anaphylaxis. It is concluded that in the complex interaction of chemical mediators of anaphylaxis in the cutaneous blood vessels of sheep, 5-HT and kinin (and/or SRS-A) may be more important than histamine.

The role of endogenous chemical mediators of anaphylactic reactions in "laboratory" animals and in man has been investigated and documented for many years. Histamine seems to be an important factor in anaphylaxis in the dog, guinea-pig and man (Code, 1937; Halpern, 1958; Humphrey & Mota, 1959). On the other hand in certain other species, histamine is thought to play a smaller part, and 5-hydroxytryptamine (5-HT) may be more important. For example, histamine antagonists have a small effect on anaphylaxis in the rat (Halpern, Liacopoulos & Perez Del Castillo 1955) and rabbit (Reuse, 1949). In mice, anaphylaxis may result in simultaneous release of histamine and 5-HT and each substance may have an approximately equal significance (Halpern, Neveu & Spector, 1963). There is little available data relating to anaphylactic reactions in the large domesticated ungulates. However, a number of disease-processes in these animals may have an anaphylactic basis; notably laminitis which occurs in the feet of all ungulates (Nilsson, 1963; McLean, 1965) "fog fever" or atypical pneumonia of cattle (Moore, 1952; Sweet 1949) bowel-oedema disease of swine (Thomlinson & Buxton, 1963) and pulmonary emphysema of horses (Andberg, 1941). Code & Hester (1939) were unable to detect histamine in the blood of horses, calves, sheep or goats during anaphylactic shock, and Alexander, Eyre & others (1969) reported that antihistamine drugs had no inhibitory effect on experimentally induced systemic anaphylaxis in sheep.

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The aim of these investigations was to study the nature of the mediators of increased capillary permeability during passive cutaneous anaphylaxis in the ungulate species using various pharmacological antagonists. This paper presents results in sheep.

EXPERIMENTAL

Materials and methods

Sheep. Either females or castrated males of the Scottish blackface and South Country Cheviot breeds were used.

Antigen. Whole dried hen egg albumin was used for sensitization, and for "challenge".

Antisera were prepared in a group of four Scottish blackface ewes using a modification of a method evolved in rabbits, described by Brocklehurst (1960). 250 mg whole ovalbumin dissolved in 5 ml isotonic saline, mixed with an equal volume of (Difco) Complete Freund's adjuvant, was injected, half subcutaneously and half intramuscularly. This was repeated after 7 days. After 6 weeks, six graded doses, 5, 10, 20, 20, 50 and 100 mg respectively, of aluminium hydroxide-absorbed ovalbumin (Colquhoun, 1964) were injected intravenously at 2-day intervals. Seven days after the last injection, 200 ml of blood was collected from each animal by venepuncture and the serum was separated, centrifuged and stored at −20°, until used.

By the P.C.A. test (see below) the most active of the four sera was determined and this was used as a standard antiserum for all tests. The antibody content was not determined.

Passive cutaneous anaphylaxis (P.C.A.). The method employed was modified from that described by Ovary (1958).

(i) *Preparation of the sheep.* At least 2 days before the beginning of experiments, the flanks and abdomens of the sheep were clipped and the areas depilated using a preparation of barium sulphide 5, detergent washing powder 1, chalk 7, and corn starch 7 parts by weight. The constituents were mixed with water to form a thick cream, applied immediately to the clipped skin and left in contact for 3–4 min. The skin was scraped free of wool with a blunt spatula, washed with warm water and toilet soap and finally dusted with talc.

(ii) *Drug injections.* A range of concentrations of each of histamine, 5-HT, bradykinin, 40/80 and serial dilutions of anti-ovalbumin sheep serum contained in 0·2 ml isotonic saline were injected intradermally, for each experiment. Four h after the intradermal injection of serum, 15 min after injection of 48/80 and immediately after the injection of histamine, 5-HT or bradykinin, sheep received 20 ml 2% Coomassie Blue and 5 ml 10% ovalbumin in isotonic saline intravenously. A period of 30 min was allowed after intravenous challenge for the full development of blue lesions at the site of intradermal injection. The diameters of the blue spots were measured with calipers and the minimum concentration of a drug or minimum dilution of serum which gave a blue spot of 1·5 cm diameter was arbitrarily taken to be the threshold dose.

The sheep were subjected to eight different regimes incorporating four antagonist drugs: mepyramine, promethazine, methysergide and sodium meclofenamate injected intravenously 15 min before "challenge". In control experiments sheep received 5 ml of isotonic saline intravenously (see Table 1).

Table 1. *The scheme of treatment of eight sheep with four antagonists of anaphylaxis: mepyramine, promethazine, methysergide and sodium meclofenamate.* Serial dilutions of histamine, 5-HT, bradykinin, compound 48/80 and serum from an ovalbumin-sensitized sheep were injected intradermally on *each* of the thirty-two occasions (see Table 2). An interval of 4 days separates "treatments" in each animal.

	Treatment with antagonists (mg/kg)			
	1	2	3	4
Sheep 1	Nil (control)	Mepyramine 5	Promethazine 5	Methysergide 1
2	Mepyramine 10	Promethazine 10	Methysergide 2	Meclofenamate Na. 1
3	Mepyramine 5	Promethazine 5	Methysergide 1	Mepyramine 10
4	Promethazine 10	Methysergide 2	Meclofenamate Na. 1	Nil (control)
5	Promethazine 5	Methysergide 1	Mepyramine 10	Promethazine 10
6	Methysergide 2	Meclofenamate Na. 1	Nil (control)	Mepyramine 5
7	Methysergide 1	Mepyramine 10	Promethazine 10	Methysergide 2
8	Melcofenamate Na. 1	Nil (control)	Mepyramine 5	Promethazine 5

The inhibitory effect of antagonists is given as the multiple of the threshold dose of agonist which is required to re-establish the 15 mm blue lesion in the presence of the antagonist (Halpern & others, 1963). This is the same concept as dose-ratio (Gaddum, Hameed & others, 1955). The mean of four measurements was calculated for each treatment.

RESULTS

Histamine

The minimum threshold dose for histamine base producing a spot diameter of 15 mm, was $0{\cdot}0025 \pm 0{\cdot}004$ μg. Mepyramine was the most powerful antagonist. 5 mg/kg reduced the activity of histamine some 3000 times and 10 mg/kg reduced histamine-activity 5000 times. Promethazine at the same dose levels was about half as active as mepyramine. Methysergide and meclofenamate each had a small but significant antihistamine action. The effect of antagonists on the thresholds of the agonists is given in Table 2.

Table 2. *The influence of the antagonists of anaphylaxis (mepyramine, promethazine, methysergide and sodium meclofenamate) on the threshold doses of histamine, 5-HT, bradykinin, compound 48/80 and antibody necessary to produce cutaneous permeability changes in the conscious sheep.* Control threshold dose of each agonist = 1 (unity) in the absence of all antagonists. Each multiple is the mean of four separate measurements in different animals (see Table 1).

Antagonist	Dose mg/kg	Multiple of threshold dose				
		Histamine	5-HT	Bradykinin	Cpd. 48/80	P.C.A.
Mepyramine	5·0	3280	1·6	—	940	14
	10·0	5020	2·9	5·0	1710	20
Promethazine	5·0	1280	8·6	—	90	156
	10·0	3520	16	—	155	382
Methysergide	1·0	9·2	255	—	50	205
	2·0	20	600	10	120	529
Sodium meclofenamate ..	1·0	40	20	100	—	505

5-Hydroxytryptamine

The threshold dose of 5-HT was 0·18 ± 0·04 μg. The data in Table 2 show methysergide to be clearly the most efficient antagonist. 1 mg/kg diminished the 5-HT response by 250 times; 2 mg/kg inhibited 600 times. The high dose of mepyramine (10 mg/kg) inhibited slightly the 5-HT responses whereas smaller doses of these drugs (5 mg/kg) had no effect. Promethazine inhibited 5-HT more strongly than mepyramine. Meclofenamate (1 mg/kg) reduced the 5-HT response by a factor of 20.

Bradykinin

The minimum dose of bradykinin to produce a 15 mm blue lesion was 0·01 ± 0·005 μg of the synthetic compound (Sandoz). Sodium meclofenamate raised the threshold a hundredfold; methysergide caused a tenfold increase and mepyramine a sixfold increase in the bradykinin threshold.

Compound 48/80

The threshold dose of compound 48/80 was 0·05 ± 0·03 μg. The antihistamine drugs were powerful antagonists of the 48/80 response. Mepyramine (5 mg/kg) reduced the activity of 48/80 by a factor of 940 and promethazine (5 mg/kg) 90 times. Doubling the dose of antihistamine approximately doubled the inhibition of 48/80. Methysergide (1 to 2 mg/kg) increased the threshold to 48/80 by 50 to 120 times.

Passive cutaneous anaphylaxis (P.C.A.)

The activity of sera from four sensitized sheep varied in ability to produce P.C.A. Threshold dilutions of the sera were as follows: Sheep 1 = 10^{-2}; Sheep 2 = 10^{-2}; Sheep 3 = 6×10^{-4}; Sheep 4 = 3×10^{-3}. The "best" titre was in Sheep No. 3 and serum from this animal was used for all subsequent tests. Intradermal injections of this serum into four unprotected control sheep gave a consistent threshold around 6×10^{-4} serum dilution.

The antihistamine agents were the poorest antagonists of P.C.A. Mepyramine at 5 mg/kg and 10 mg/kg increased the threshold dose of antibody only slightly—namely 14 times and 20 times respectively. Promethazine at 5 mg/kg and 10 mg/kg was more potent, producing increases in threshold dilution from 156 to 382 times respectively. Methysergide was more potent than the antihistamine drugs. A dose of 2 mg/kg of methysergide increased the threshold of serum dilution some 500 times. Sodium meclofenamate similarly reduced the anaphylactic reaction of the skin vessels by a factor of 500.

DISCUSSION

A total of eight sheep were used and a scheme of treatments devised to allow four experiments per animal, each procedure separated by a 4-day interval. This method introduces potential problems associated with persistence of drugs namely the dye substance, the antigen and the antagonist.

Coomassie Brilliant Blue (George T. Gurr and Co. Ltd., London, N.W.9) was used throughout because it has been shown to be non-persistant (Feinberg & Dewdney, 1963). Coomassie Blue did not persist in the skin or subcutis of sheep for longer than 48 h.

There did not seem to be any disadvantage due to the possible persistence of antigen in circulation. Although some antibodies would undoubtedly be produced

during the 12-day period of exposure to antigen, there appeared to be no marked interference with the formation of distinct P.C.A. reactions. Three animals developed mild transient dyspnoea following protein injection on the 12th day.

Sheep do not readily show anaphylactic reactions within a 2-week period of "simple" protein injections as described here. This species seems to require a more extended and sophisticated regime for protein sensitization, including the use of adjuvants (e.g. Freund's) or "boosting" with alum-precipitated protein, or both, before being capable of showing a marked systemic anaphylaxis (Alexander & others, 1969). This is in contrast with cattle which readily become sensitized and show a severe reaction within 7 days of a single sensitizing dose of protein (Aitken & Sanford 1969).

It appears from the data that the sensitivity of the peripheral blood vessels of sheep to histamine is greater than in the laboratory species. The threshold dose in sheep was 0·0025 μg whereas in guinea-pigs it is 0·3 μg, in the rat 0·9 μg and in the mouse 0·15 μg (Halpern & others, 1963).

The sensitivity of sheep capillaries to 5-HT is about 70 times less than to histamine whereas the sensitivity to bradykinin only four times smaller.

The effectiveness of the antagonist drugs in sheep appears to be qualitatively similar to that in other species. Mepyramine and promethazine antagonized histamine strongly whereas methysergide and meclofenamate had much less antihistamine activity. Good correlation existed between the other active substances and their antagonists. 5-HT was inhibited by methysergide whereas the antihistamine drugs and meclofenamate had but a small effect on 5-HT.

Compound 48/80 was strongly inhibited by mepyramine and weakly by promethazine and methysergide. It is probable that compound 48/80 acts principally by releasing histamine but the inhibition by methysergide suggests that compound 48/80 may liberate some 5-HT in sheep, as has been shown in rats (Bhattacharya & Lewis, 1956). It would be of interest to clarify this point by direct determination.

Passive cutaneous anaphylaxis is well inhibited by meclofenamate and methysergide but relatively poorly by the antihistamine drugs. Thus it is unlikely that peripheral vascular permeability changes as a result of local anaphylaxis are due solely to the liberation of histamine in sheep. If histamine were the principle mediator of the anaphylactic response, one would expect powerful inhibition of mepyramine since mepyramine was shown simultaneously to inhibit strongly the actions of histamine itself on skin vessels in sheep.

On the other hand methysergide, 1 to 2 mg/kg, strongly inhibits 5-HT (threshold increased from 250–600 times) and the P.C.A. reaction is similarly reduced 530-fold. This contrasts with the weak inhibition of histamine and bradykinin by methysergide (9 to 20-fold reduction). This specificity of methysergide for 5-HT suggests that the amine is involved in cutaneous anaphylaxis in the sheep. It is further interesting that promethazine which exhibits some anti-5-HT activity, is intermediate between mepyramine and methysergide in inhibiting the P.C.A. reaction.

Sodium meclofenamate was the least specific antagonist showing some inhibition of all the active agents, although the inhibition by meclofenamate of bradykinin was at least double that of either histamine or 5-HT. Meclofenamate has been shown to be a powerful antagonist of bradykinin, SRS and antigen induced bronchoconstriction in the guinea-pig (Berry & Collier, 1964; Collier & James, 1967; Collier, James & Piper, 1968), but this antagonist is less effective in inhibiting the action of kinins on blood vessels than on bronchial muscle.

Meclofenamate and methysergide each inhibited the P.C.A. reaction to approximately the same extent. Alexander & others (1969) investigated the protection produced against experimental general anaphylaxis in sheep by mepyramine methysergide and meclofenamate. These authors described meclofenamate as the best antagonist whereas mepyramine and methysergide afforded little protection on the cardiovascular and respiratory systems. Meclofenamate antagonized the effects of exogenously administered bradykinin in sheep.

Evidence for the participation of various mediators has thus been obtained indirectly; there being no direct estimation of active agents. Nevertheless the establishment simultaneously of the specificity of each antagonist makes it possible to postulate the relative importance of each potential mediator and allows the conclusion that cutaneous anaphylaxis in sheep is mediated by the interaction of histamine, 5-HT and kinin; with the possible addition of SRS-A and other substances. Kinin and 5-HT appear to be more important than histamine in these circumstances, but it may not be valid to extend these conclusions to other sites or to generalized systemic anaphylaxis, where the relative importance of the mediators may well differ.

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Neuromuscular blocking agents. Replacement of quaternary ammonium groups in bis-onium compounds by amidinium, guanidinium, thiouronium, sulphonium and sulphoxonium groups

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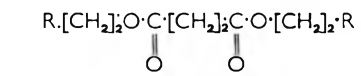
The quaternary ammonium groups of suxamethonium have been replaced by amidinium, guanidinium and thiouronium groups. The guanidine compound exhibited competitive neuromuscular activity of a low order of potency; the other compounds were ineffective. Replacement of the quaternary ammonium groups in decamethonium by ethylmethylsulphonium or dimethylsulphoxonium groups gave less potent, depolarizing agents.

The primary chemical requirement for a neuromuscular blocking agent, according to Stenlake (1963), is a strongly basic centre capable of permanent existence as a positively charged ion. While in most active compounds this basic centre is a quaternary ammonium group, blocking properties have been demonstrated for other onium compounds such as sulphonium, phosphonium, arsonium and stibonium compounds, certain tertiary amines, and guanidine derivatives.

As part of a program aimed at the development of a short-acting, competitive, blocking drug, we have investigated the effect on the nature and duration of activity of suxamethonium (Ia) and decamethonium (IIa) brought about by replacement of the quaternary ammonium groups by other strongly basic groups.



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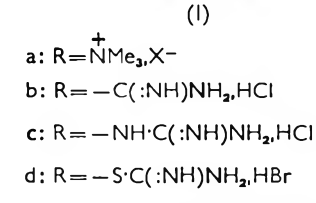
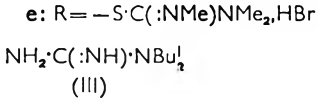
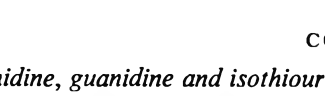
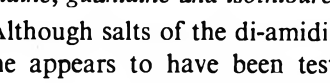
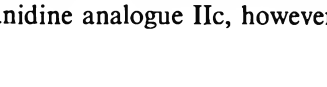




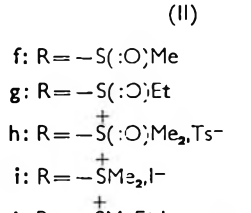


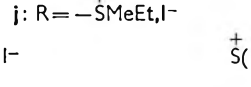


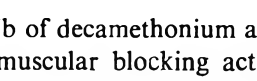


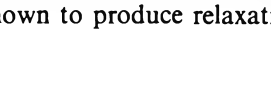
(III)

(II)









(IV)

(V)

COMPOUNDS STUDIED

Amidine, guanidine and isothiourea derivatives

Although salts of the di-amidine analogue IIb of decamethonium are well known, none appears to have been tested for neuromuscular blocking activity. The di-guanidine analogue IIc, however, has been shown to produce relaxation in the frog

sciatic-sartorius muscle preparation which was antagonized by potassium chloride and neostigmine (Ozawa, Fukuda & Goto, 1962, Ozawa, Gomi & Watanabe, 1964) Certain *NN*-disubstituted guanidines have been found to cause flaccid paralysis in fowls, cats, mice and rabbits which was not antagonized by cholinesterase inhibitors (Barzaghi, Mantegazza & Riva, 1965). One of the most active compounds was *NN*-di-isobutylguanidine (III), which was more active than gallamine triethiodide in mice, but less active in the rabbit.

The di-isothiourea analogues IId and IIe of decamethonium produced about 1/30 the activity of tubocurarine in the rabbit head-drop test and 1/30 and 1/100 the activity of tubocurarine respectively in the frog rectus abdominis muscle preparation (Cheymol, Chabrier & others, 1953).

Preparation of the di-amidine (Ib), di-guanidine (Ic) and di-isothiourea (Id) analogues of suxamethonium was therefore undertaken.

Sulphonium and sulphoxonium salts

The di-sulphonium compound IIi has been found to be approximately one-third as active as gallamine triethiodide in the rabbit head-drop test for neuromuscular blocking activity (Walker, 1950).

Major & Hess (1958) demonstrated in dogs that trimethylsulphoxonium iodide (IV) showed some of the muscarinic and nicotinic properties of acetylcholine, but the compound was only 1/1000 to 1/10,000 as active as acetylcholine in lowering the blood pressure and its nicotinic activity was less than that of acetylcholine.

The possibility that analogous di-sulphoxonium salts such as IIh might exhibit short-acting neuromuscular blocking activity was suggested by our colleague Dr. R Slack. That salt was therefore examined and the intermediate compound used in its preparation was also used to prepare a higher homologue (IIj) of the di-sulphonium salt (IIi).

CHEMISTRY

Condensation of 2-amidinoethanol or 2-guanidinoethanol with succinyl chloride at 130° gave respectively di-2-amidinoethyl succinate dihydrochloride (Ib) or di-2-guanidinoethyl succinate dihydrochloride (Ic) in low yield. Di-2-amidinothioethyl succinate dihydrobromide (Id) was obtained in moderate yield from di-2-bromoethyl succinate and thiourea in boiling ethanol. For all three compounds the structures assigned were supported by the presence of ester and amidine bands in the infrared spectra.

Decamethylenebis(ethylmethylsulphonium iodide) (IIj) was obtained directly from 1,10-di(methylthio)decane and ethyl iodide at room temperature. Attempts to ethylate 1,10-di(ethylthio)decane with ethyl iodide to give a higher homologue were not successful.

Kuhn (1957) and Kuhn & Trischmann (1958) found that dimethyl sulphoxide and methyl iodide react to give the stable sulphoxonium salt IV. However, Smith & Winstein (1958) showed that the action of dimethyl sulphoxide on reactive halides or esters can give rise to two different types of derivative, the *S*-alkyl compound such as IV or the *O*-alkyl compound such as V. The unstable *O*-alkyl derivatives tend to isomerize in solution to the stable *S*-alkyl adducts, which are the normal reaction products from a sulphoxide with methyl iodide or an alkyl toluenesulphonate (cf. also Natus & Goethals, 1965).

1,10-Di(methylsulphinyl)decane (IIf) has been described by Jerchel, Dippelhofer & Renner (1954), who obtained it by oxidation of 1,10-di(methylthio)decane with hydrogen peroxide in acetic acid. It was conveniently prepared under conditions where sulphone formation is negligible by periodate oxidation of the sulphide, the same method also being employed for the preparation of the corresponding ethyl compound IIg.

Methylation of 1,10-di(methylsulphinyl)decane was achieved in low yield by prolonged heating at 120° with methyl toluene-*p*-sulphonate to give the di-sulphoxonium tosylate IIh. Attempted ethylation of either IIf or its ethyl homologue IIg with ethyl toluene-*p*-sulphonate or ethyl 2,4-dinitrobenzenesulphonate was not successful.

PHARMACOLOGICAL METHODS

Cats were anaesthetized with ether followed by chloralose (80–100 mg/kg) intravenously. The tibialis muscle of one leg was prepared for recording responses to supramaximal stimulation of the sciatic nerve as described by Bamford, Biggs & others (1967). Drugs were injected via a polythene cannula inserted into a femoral vein. Dose response lines were constructed for each compound where possible. Mechanism of action was investigated (i) by attempting to reverse neuromuscular blockade with edrophonium (0·5 mg/kg) intravenously and (ii) by intravenous injection of each compound into day-old chicks.

The intravenous LD50 of each compound was determined in mice, and mortalities were observed for 24 h.

The drugs used were: α-chloralose (Koch–Light Labs.), decamethonium iodide, edrophonium chloride ("Tensilon", Roche Products Ltd.), gallamine triethiodide ("Flaxedil", May & Baker Ltd.) and suxamethonium bromide ("Brevidil M", May & Baker Ltd.). All doses refer to the appropriate salt of each compound.

Table 1. *Analogues of suxamethonium*

$R{\cdot}[CH_2]_2{\cdot}O{\cdot}CO{\cdot}[CH_2]_2{\cdot}CO{\cdot}O{\cdot}]CH_2]_2{\cdot}R$

Compound	R	LD50 in mice mg/kg intravenously	Blocking dose on cat sciatic nerve-tibialis muscle preparation ED50 mg/kg intravenously	Duration of action of ED50 in min	Mechanism of action
Ia (Suxamethonium)	$-\overset{+}{N}Me_3,\ Br^-$	0·71	0·032	4·5	Depolarizing
Ib	$-C(:NH){\cdot}NH_2,HCl$	*ca* 100	>40·0	—	—
Ic	$-NH{\cdot}C(:NH){\cdot}NH_2,HCl$	*ca* 75	10% at 30·0	—	Competitive
Id	$-S{\cdot}C(:NH){\cdot}NH_2,HBr$	*ca* 100	>40·0	—	—
Gallamine triethiodide	—	48	0·68	18	Competitive

RESULTS AND DISCUSSION

Di-amidine, -guanidine and -isothiourea analogues of suxamethonium (Table 1)

Only the di-guanidine analogue Ic showed any neuromuscular blocking activity at the dose levels used, but it was much less active than either gallamine triethiodide or suxamethonium.

This observation suggests that potency in this series is related to basic strength. The following values of pK_a have been reported (Perrin, 1965): guanidine, 13·6;

N-methylguanidine, 13·4; amidinomethane (acetamidine), 12·1, 12·4; *S*-methylisothiourea, 9·78, 9·81. Although a strong base, the diguanidine analogue Ic would be weaker than suxamethonium, which as a quaternary ammonium salt must necessarily be completely ionized (Albert, 1968).

Table 2. *Analogues of decamethonium*

$R{\cdot}[CH_2]_{10}{\cdot}R$

Compound	R	LD50 in mice mg/kg intravenously	Blocking dose on cat sciatic nerve-tibialis muscle preparation ED50 mg/kg intravenously	Duration of action of ED50 in min	Mechanism of action
IIa (Decamethonium)	$-\overset{+}{N}Me_3,I^-$		0·008	18	Depolarizing
IIf	$-S(:O)Me$	37·5	> 7·0	—	—
IIg	$-S(:O){\cdot}Et$	>30	>10·0	—	—
IIh	$-\overset{+}{S}(:O){\cdot}Me_2,Ts^-$ *	*ca* 10	1·1	12	Depolarizing
IIj	$-\overset{+}{S}(Me){\cdot}Et,I^-$	12·8	0·55	16	Depolarizing

* Ts = toluene-*p*-sulphonate.

Sulphonium and sulphoxonium analogues of decamethonium (Table 2)

The uncharged sulphoxides IIf and IIg were devoid of activity at the dose levels used, but both the disulphoxonium salt IIh and disulphonium salt IIj showed activity on the cat nerve-muscle preparation. Both compounds were much less effective than decamethonium, but had potencies of the order of that of gallamine triethiodide. Both compounds had a depolarizing mechanism of action. A typical experiment with compound IIj is shown in Fig. 1.

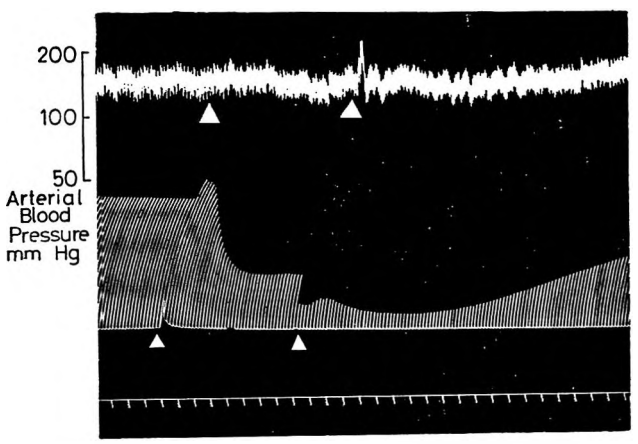


FIG. 1. Cat sciatic nerve–tibialis muscle preparation. At the first mark compound IIj (0·375 mg/kg, intravenously) was injected. This caused twitch potentiation followed by a neuromuscular blockade which was potentiated by injection of edrophonium (0·5 mg/kg, intravenously) at the second mark. Time scale in min.

It was noteworthy that the di(ethylmethylsulphonium) salt IIj was approximately equipotent with gallamine triethiodide in the cat (see Table 1), for in the rabbit the di(dimethylsulphonium) salt IIi had only approximately one-third the activity of gallamine triethiodide (Walker, 1950). Replacement of one methyl group by an ethyl group therefore seems to have increased neuromuscular blocking activity whereas in decamethonium itself this change decreases potency (Barlow, 1964).

EXPERIMENTAL

Di-2-amidinothioethyl succinate dihydrobromide. A mixture of di-2-bromoethyl succinate (Fusco, Palazzo & others, 1949) (8·3 g, 0·025 mol) and thiourea (3·8 g, 0·05 mol) was heated under reflux for 5 h in ethanol (50 ml). Ethyl acetate was added to produce incipient turbidity, and the mixture was then chilled. The separated material (6·1 g, m.p. 164–167°) was crystallized from ethanol-ether to give the *di-isothiourea* derivative (5·3 g, 44%) as colourless needles, m.p. 166–167° (Found: N, 11·6; Br, 32·7. $C_{10}H_{18}N_4O_4S_2$,2HBr requires N, 11·6; Br, 33·0%).

Di-2-guanidinoethyl succinate dihydrochloride. 2-Guanidinoethanol hydrochloride (Beatty & Magrath, 1960) (1·4 g, 0·01 mol) was mixed with succinyl chloride (2 ml, 0·005 mol) whilst cooling in ice, and the mixture was then heated in an oil bath at 130° until a clear viscous solution formed. This was shaken with a mixture of acetone and ether, and the solvents were decanted from a pasty residue, which became solid on treatment with ethanol, and was filtered off and washed with ether. Recrystallization of the product (0·61 g, m.p. 167–170°) from ethanol-ether gave the *di-guanidine hydrochloride* (0·48 g, 14%) as a colourless powder, m.p. 168–170° (Found: N, 22·9; Cl, 19·8. $C_{10}H_{20}N_6O_4$,2HCl requires N, 23·3; Cl, 19·6%).

Similarly 2-amidinoethanol (Price & Zomlefer, 1949) (4·15 g, 0·033 mol) and succinyl chloride (2·6 g) gave *di-2-amidinoethyl succinate dihydrochloride* (0·65 g, 12%) as a colourless powder, m.p. 153–154° (Found: C, 36·5; H, 6·4; Cl, 21·6. $C_{10}H_{18}N_4O_4$,2HCl requires C, 36·3; H, 6·1; Cl, 21·4%).

Decamethylenebis(ethylmethylsulphonium iodide). A mixture of 1,10-di(methylthio)decane (Walker, 1950) (0·234 g, 0·001 mol) and ethyl iodide (3 ml) was allowed to stand at room temperature for 3 days. Anhydrous ether was added, and the oil thus formed slowly solidified. Crystallization of the solid from acetone gave the *salt* (0·33 g, 60%) as colourless needles, m.p. 110–111° (Found: S, 11·5; I, 46·1. $C_{16}H_{36}I_2S_2$ requires S, 11·7; I, 46·5%).

1,10-Di(ethylthio)decane, prepared (61%) in an impure state by the method of Walker (1950), had b.p. 195–197°/15 mm. (Found: C, 64·9; H, 11·8; S, 23·6. Calc. for $C_{14}H_{30}S_2$: C, 64·1; H, 11·4; S, 24·4%), and was oxidized directly (see below).

1,10-*Di(methylsulphinyl)decane.* The procedure of Leonard & Johnson (1962) was employed. 1,10-Di(methylthio)decane (2·34 g, 0·01 mol) in methanol (100 ml) was added with stirring to sodium metaperiodate (42 ml of a solution made by dissolving 5·35 g in 50 ml water) (0·021 mol), and the mixture stirred vigorously at 0° for 12 h. The solution was filtered, and the residue remaining after removal of the methanol was extracted thrice with 50 ml portions of boiling chloroform. Removal of the chloroform gave a colourless solid (1·9 g, m.p. 108–110°), which was recrystallized from benzene to give the sulphoxide (1·6 g, 60%) as colourless leaflets, m.p. 110–112° (Found: C, 53·9; H, 9·6; S, 24·2. Calc. for $C_{12}H_{26}O_2S_2$: C, 54·1; H, 9·8; S, 24·1%). Jerchel & others (1954) give m.p. 119–120°.

Similarly 1,10-di(ethylthio)decane (2·62 g, 0·01 mol) gave 1,10-*di(ethylsulphinyl)decane* (2·3 g, 78%) as colourless needles from benzene, m.p. 108–109° (Found: C, 57·4; H, 10·3; S, 21·9. $C_{14}H_{30}O_2S_2$ requires C, 57·1; H, 10·3; S, 21·7%).

Decamethylene bis(dimethylsulphoxonium toluene-p-sulphonate). A mixture of 1,10-di(methylsulphinyl)decane (1·33 g, 0·005 mol) and methyl toluene-*p*-sulphonate (1·86 g, 0·01 mol) was heated in an oil bath at 120° for 96 h. The reaction mixture was triturated with acetone and filtered. The brown solid residue (0·09 g, m.p.

235–238°) was crystallized from ethanol-ether (charcoal) to give the salt (0·07 g, 2·2%) as a colourless powder, m.p. 238–240° (Found: C, 52·8; H, 7·2; S, 20·2 $C_{28}H_{46}O_8S_4$ requires C, 52·6; H, 7·3; S, 20·1%).

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Pharmacological screening of some Brazilian plants

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Forty-five species of plants from several botanical families growing in North East Brazil have been examined for toxicity on mice and small fishes, cat blood pressure and respiration, isolated toad heart and rectus abdominis muscle, guinea-pig ileum, rabbit duodenum and rat uterus. A high toxicity to mice and fishes was exhibited by aqueous (A) and ethanolic (B) extracts from *Luffa operculata*, *Peschiera affinis*, *Pithecelobium multiflorum* (gall) and *Zizyphus joazeiro* and by extract B from *Pithecelobium multiflorum* (stem-bark). Cardiorespiratory activity was shown by *Annona squamosa*, *Byrsonima sericea*, *Crataeva tapia*, *Erythrina velutina*, *Fagara rhoifolia*, *Operculina macrocarpa*, *Perschiera affinis*, *Pithecelobium multiflorum*, *Spondias lutea* and *Zizyphus joazeiro*. Extracts A and B from *Operculina macrocarpa* and *Pithecelobium multiflorum*, extract A from *Luffa operculata* and *Zizyphus joazeiro* and extract B from *Crataeva tapia* and *Peschiera affinis* promoted a contraction of the toad rectus abdominis muscle. Both extracts from *Annona aquamosa* and *Fagara rhoifolia* (leaf) provoked a spasmogenic effect on guinea-pig ileum and a spasmolytic one on rabbit duodenum. Extracts A and B from *Pithecelobium multiflorum*, *Vitex gardneriana* and *Zizyphus joazeiro* exhibited a spasmogenic activity on both preparations, while extracts A and B from *Peschiera affinis* and extract B from *Erythrina velutina* also evidenced a spasmolytic activity on both preparations. Oxytocic activity was shown by both extracts from *Annona squamosa*, *Byrsonima sericea*, *Pithecelobium multiflorum* and *Vitex gardneriana*.

We have made a pharmacological examination of forty-five plant species growing in the neighbourhood of Fortaleza (Brazil) along similar lines to those adopted by Feng, Haynes & others (1962) in their examination of West Indian plants.

The tests were arranged according to Laurence & Bacharach (1964) and Turner (1965). The work was aimed at selecting interesting material for further study.

EXPERIMENTAL

Plant extractions

Botanically identified species were collected and processed immediately. Extracts were prepared by boiling the reduced part of the plant, on a water bath, in distilled water (extract A) or ethanol (extract B) filtering through calico and repeating the procedure with the residue. The filtrates were combined and evaporated. The final concentration of the extracts were adjusted to give the equivalent of 1 g material per ml of solvent. Where the viscosity was unsuitable for pharmacological experiments the concentration was adjusted to 1:10. Before use the ethanolic extract was evaporated on a water bath and its original volume made up with distilled water and neutralized with N sodium hydroxide when necessary and stored at 4°.

Pharmacological testing

The following tests were made on the extracts.

Acute toxicity in mice. Extracts A and B were given by intraperitoneal injection (0·1, 0·2, 0·4 and 0·8 ml) to groups of four adult mice of either sex and random strain. The minimum dose required to kill all animals of a group within 24 h was used as the toxic dose level (Table 1).

Acute toxicity in the fish Lebistes reticulatus. Ten fishes were placed in flasks containing 0·5 ml of the extracts in 300 ml of water and the lethality (%) after 24 h recorded. Water or water plus 0·5 ml of ethanol was used as a blank.

Cat respiration and blood pressure. Adult cats anaesthetized with pentobarbitone sodium (30 mg/kg, i.p.) were prepared for the measurement of respiratory movements and carotid blood pressure after being given heparin. After slow injection of extract (0·5–2·0 ml) through the femoral vein, pressor or depressor responses and stimulation or depression of respiration were noted (Table 1).

Toad rectus abdominis muscle preparation (Burn, 1952; Valle, 1955). Extracts (0·1–0·5 ml) enhancing the contraction of the muscle or antagonizing acetylcholine-induced contractions (up to 20 μg) were recorded (Table 1).

Toad heart preparation. The effects on heart rate, amplitude and rhythm were registered. Injections of extracts (0·1–0·5 ml) were made by a syringe through the polyethylene tube on the side arm of the perfusion cannula, containing 5 ml of frog-Ringer solution, tied in the inferior vena cava.

Guinea-pig ileum and rabbit duodenum in vitro. Pieces of gut were set up in a 10 ml bath containing aerated Tyrode solution at 37° to record the effect up to 0·5 ml of the extracts on the longitudinal muscle contractions (Burn, 1952). Acetylcholine (up to 10 μg) was used as control drug. The effects of extracts (0·1–0·5 ml) on tonus and motility of the rabbit duodenum were also noted (Table 1).

Rat uterus. A segment of uterus from an adult virgin rat pretreated with 10 μg of stilboestrol was suspended in a 10 ml bath containing the De Jalon solution and longitudinal contractions were recorded in the usual way. Oxytocin (0·001–0·01 i.u.) was used as control. The extracts (0·1–0·5 ml) were tested for their spasmogenic effect and also their ability to inhibit contractions induced by oxytocin.

RESULTS

The results are tabulated in Table 1. Among the species of plants we have studied a high level of toxicity was shown by extracts from *Krameria tomentosa* (root), *Luffa operculata*, *Peschiera affinis*, *Pithecelobium multiflorum* (gall), *Simaruba versicolor* (root-bark), *Triplaris gardneriana* and *Zizyphus joazeiro*. Whereas most extracts exhibited a depressor action upon the carotid blood pressure only the ethanolic extract of *Borreria verticilata* (overground portion) and *Fagara rhoifolia* and aqueous and ethanolic extract from *Delonix regia* exhibited a pressor action.

Nearly all the species studied were active on the cat respiration, and have stimulant or depressor effects.

Contraction of the toad rectus abdominis muscle was observed mainly after addition to the bath of aqueous and ethanolic extracts from *Operculina macrocarpa*, *Pithecelobium multiflorum* and *Zizyphus joazeiro*, aqueous extract from *Luffa operculata* and *Sapindus saponaria* and ethanolic extract from *Crataeva tapia*.

Table 1. *Pharmacological actions of aqueous extracts (A) and ethanolic extracts (B) of forty-five Brazilian plants*

Species, family, common usage name	Part used	Extract and concn used		Toxicity		Cat		Toad		G.P.	Rabbit duodenum		Rat
				Mice	Fish	B.P.	Resp.	Rect. abd.	Heart	ileum	Tonus	Motility	uterus
Alpinia speciosa Schum.	Leaf	A	1:1	O	O	O	O	O	D	+++	−	O	++
Zingiberaceae (Colônia)		B	1:1	O	O	O	O	+	D	−	−	−	−
Anacardium occidentale L.	Tegument	A	1:3	O	100	D	D	O	D	+	−	O	+++
Anacardiaceae (Cajueiro)		B	1:1	†	100	D	S	++	D	+	−	O	−
Annona squamosa L.	Leaf	A	1:10	†††	O	D	S	O	S	+++	−	−	+++
Annonaceae (Ateira, Ata)		B	1:1	††	O	D	S	−	D	+++	−	−	+++
Boerhaavia coccinea Mill.	Root	A	1:1	†	O	O	S	O	S	+	+	O	+
Nyctaginaceae (Pega-pinto)		B	1:1	O	O	O	S	O	S	O	−	O	+
Borreria verticilata J. F. W. Mayer	Root	A	1:1	O	O	D	S	O	D	O	+	+	++
Rubiaceae (Vassourinha de botão)		B	1:1	O	O	D	S	O	D	O	+	−	++
	Overground portion	A	1:3	O	O	D	S	−	D	++	−	−	+++
		B	1:1	O	O	D	S	−	D	++	−	−	+++
Byrsonima sericea D.C.	Stem-bark	A	1:10	††	100	D	D	O	S	−	+	O	+++
Malpigulaceae (Murici-pitanga)		B	1:10	††	100	D	D	O	S	−	+	−	+++
Cannabis sativa L. ♀.	Flowering tops	A	1:2	†††	O	O	O	O	D	+	−	−	+++
Cannabinaceae (Maconha)		B	1:2	O	100	O	S	O	D	−	−	−	+++
Cecropia carbonaria Mart.	Leaf	A	1:1	†	O	D	O	O	D	−	−	−	+
Moraceae (Torém, Gargaúba)		B	1:1	†	O	D	O	O	D	−	−	−	++
Cochlospermum insigne St. Hil.	Stem-bark	A	1:2	††	O	O	O	O	D	++	−	−	+++
Cochlospermaceae (Pacotê)		B	1:1	O	O	O	O	O	D	−	−	O	+++
Cochlospermum vitifolium Spreng.	Stem-bark	A	1:1	†††	O	O	O	O	D	++	−	+	+++
Cochlospermaceae (Algodão-bravo)		B	1:1	O	O	O	O	O	D	++	+	−	++
Coutarea hexandra Schum.	Stem-bark	A	1:1	†	10	D	O	−	D	+++	+	−	−
Rubiaceae (Quina-quina)		B	1:1	†	10	D	O	−	D	−	−	−	−
Crataeva tapia L.	Leaf	A	1:5	O	O	D	S	+	D	+++	−	−	+++
Capparidaceae (Trapiá, tapiá)		B	1:5	O	O	D	S	+++	D	+++	+	−	O
	Stem-bark	A	1:1	O	O	D	S	+++	D	+++	−	O	O
		B	1:1	O	O	D	S	+++	D	+++	O	−	O
Curatella americana L.	Stem-bark	A	1:2	†††	O	D	O	−	S	O	−	O	−
Dilleniaceae (Cajueiro-bravo)		B	1:1	††	100	D	O	−	S	+	−	−	−
Delonix regia Raf.	Stem-bark	A	1:1	†††	O	D	P	O	S	+++	−	−	−
Leguminosae papilionoideae (Flambuaiã)		B	1:1	†	O	D	S	O	D	+++	−	O	++
Diodia barbeyana Hub.	Root	A	1:1	O	O	D	O	−	S	−	O	−	+
Rutaceae (Saca-cstrepe)		B	1:1	O	O	D	D	O	S	−	−	−	−
Erythrina velutina Wild.	Stem-bark	A	1:1	O	O	D	S	−	D	+	−	−	+++
Leguminosae papilionoideae (Mulungu)		B	1:1	†	10	D	D	−	D	−	−	−	++
Fagara rhoifolia Engl.	Leaf	A	1:1	†	10	D	D	O	S	+++	−	−	−
Rutaceae (Limãozinho)		B	1:1	†	O	D	O	O	S	+++	−	−	−
	Stem-bark	A	1:1	O	O	D	O	O	S	O	−	−	−
		B	1:1	†	O	D	O	++	S	+++	−	−	−
Guazuma ulmifolia Lam.	Stem-bark	A	1:1	†	O	O	O	O	D	−	−	−	+
Sterculiaceae (Mutamba)		B	1:1	O	O	O	O	+	S	−	−	−	++
Guetarda angelica Mart.	Root-bark	A	1:2	†	O	O	D	O	S	−	−	O	++
Rubiaceae (Angélica)		B	1:1	††	O	O	D	O	S	−	+	−	++
Heliotropium indicum L.	Root	A	1:1	†	O	O	D	O	S	O	+	−	+++
Borraginaceae (Fedegoso)		B	1:1	O	O	O	D	O	S	−	−	O	+++
Hymenaea courbaril L.	Stem-bark	A	1:2	†††	O	O	D	O	S	O	+	−	−
Leguminosae papilionoideae (Jatobá)		B	1:2	†	100	D	D	−	D	−	−	O	−
Krameria tomentosa Mart.	Stem	A	1:10	†††	O	D	D	O	O	O	+	−	O
Leguminosae caesalpinoideae (Carrapicho)		B	1:10	††	20	D	D	O	O	−	+	O	O
	Leaf	A	1:10	††	100	D	D	O	S	−	+	−	O
		B	1:10	††	100	D	D	O	O	−	+	O	+

Table 1—*continued*

Krameria tomentosa	Root	A	1:1	†††	100	O	O	O	O	--	O	O	-
		B	1:1	†††	100	D	S	O	D	---	O	O	---
Lagancularia racemosa Gaert.	Leaf	A	1:1	†††	70	D	S	O	D	-	-	-	-
Combretaceae (Mangue branco)		B	1:1	†††	60	D	S	O	D	--	-	-	-
Lantana camara L.	Root	A	1:1	††	O	D	S	O	S	O	+	O	O
Verbenaceae (Camará)		B	1:1	†	O	O	O	O	S	O	+	O	O
Luffa operculata Cogn.	Fruit	A	1:10	†††	90	O	S	+++	D	+	+	-	+
Cucurbitaceae (Cabacinha)		B	1:10	†††	100	O	S	+	D	+	+	-	+
Operculina macrocarpa Urban.	Tuber	A	1:2	†††	O	D	D	+++	D	++	+	-	++
Convolvulaceae (Batata de purga)		B	1:2	†††	O	D	D	+++	D	++	+	-	++
Peschiera affinis Miers.	Root-bark	A	1:1	†††	100	D	S	--	D	--	-	-	+
Apocinaceae (Grão-de-galo)		B	1:1	†††	100	D	S	+++	D	--	-	-	+
Pithecelobium multiflorum Benth.	Stem-bark	A	1:1	†††	O	D	S	+++	D	+++	+	-	++
Leguminosae mimosoideae (Canafistula de boi)		B	1:1	†††	100	D	S	+++	D	++	+	-	+++
	Gall	A	1:2	†††	100	D	O	+++	D	+++	+	-	+++
		B	1:2	†††	100	D	O	+++	D	+++	+	-	+++
Plumeria bracteata A.D.C.	Root-bark	A	1:1	††	100	O	O	O	S	O	-	O	+
Apocinaceae (Janaguba)		B	1:1	O	100	O	O	O	S	O	-	O	+
Sapindus saponaria L.	Stem-bark	A	1:1	O	O	D	D	+++	D	+++	+	-	--
Sapindaceae (Sabonete)		B	1:1	O	O	O	O	O	D	+	+	O	---
Scoparia dulcis L.	Root	A	1:1	††	O	D	D	O	S	O	-	-	+
Scrophulariaceae (Vassourinha)		B	1:1	O	O	D	D	O	S	O	-	-	+
Simaruba versicolor St. Hill.	Stem-bark	A	1:5	†††	O	D	D	--	D	---	-	-	---
Simarubaceae (Pau paraíba)		B	1:2	†	O	D	O	---	D	---	-	-	---
	Root-bark	A	1:1	†††	100	D	O	++	D	-	-	O	+
		B	1:1	†††	90	D	O	+	D	--	-	-	++
Solanum paniculatum L.	Root	A	1:1	††	O	D	S	+	S	-	-	-	+
Solanaceae (Jurubeba)		B	1:1	†††	90	D	D	+	S	--	-	-	+
Spondias lutea L.	Stem-bark	A	1:1	†	O	D	D	++	D	++	+	O	++
Anacardiaceae (Cajazeira)		B	1:1	†	100	D	D	O	D	++	+	+	+++
Strychnos parvifolia D.C.	Fruit	A	1:1	†	O	D	D	O	S	-	-	-	-
Loganiaceae (Urari)		B	1:1	O	O	D	D	O	S	-	-	-	-
	Seed	A	1:1	†	O	D	D	O	S	++	-	+	O
		B	1:1	O	O	D	D	O	S	-	-	+	-
Tabebuia caraiba Mart.	Stem-bark	A	1:1	††	O	D	O	++	D	-	-	-	---
Bignoniaceae (Caraúba)		B	1:1	††	O	D	D	O	D	---	-	-	---
Talisia esculenta Radlk.	Stem-bark	A	1:1	†	O	D	S	O	D	++	-	O	O
Sapindaceae (Pitombeira)		B	1:1	O	O	D	O	O	O	++	O	O	O
Tapirira guyanensis Aubl.	Stem-bark	A	1:1	†††	100	D	D	+	S	O	+	O	++
Anacardiaceae (Pau-pombo)		B	1:2	O	100	D	D	-	S	O	+	O	+
Tocoyema formosa Schum.	Root-bark	A	1:1	†	O	D	D	O	D	O	+	O	+++
Rubiaceae (Jenipapo-bravo)		B	1:1	†	O	O	O	O	D	O	-	O	--
Triplaris gardneriana Wild.	Stem-bark	A	1:2	††	100	D	D	O	S	+	+	O	++
Poligonaceae (Pajeú)		B	1:2	†††	100	D	O	O	S	++	+	O	+++
Turnera sp.	Root	A	1:1	†	O	D	S	O	D	-	-	-	+
Turneraceae (Chanana)		B	1:1	O	O	D	S	O	D	O	-	O	+++
Vitex gardneriana Schau.	Stem-bark	A	1:1	†	O	O	O	++	D	+++	+	-	+++
Verbenaceae (Tarumã)		B	1:1	O	O	D	D	O	D	+++	+	+	+++
Wilbrandia sp.	Tuber	A	1:1	††	100	O	O	-	S	-	+	+	+
Cucurbitaceae (Cabeça-de-negro)		B	1:1	††	70	D	D	-	S	-	-	-	-
Zizyphus joazeiro Mart.	Stem-bark	A	1:1	†††	100	D	S	+++	S	+++	+	+	+
Ramnaceae (Juàzeiro)		B	1:1	†††	100	D	S	++	S	+++	+	-	++
Zizyphus undulata Reiss.	Stem-bark	A	1:1	††	40	D	S	++	D	+++	+	+	+++
Ramnaceae (Juamirim)		B	1:1	†	O	O	O	O	S	++	-	O	++

A = aqueous extract
B = ethanolic extract
† = degree of toxicity

number = death per cent
D = depressor effect
P = pressor effect

S = stimulant effect
\+ = stimulant effect
− = inhibition.

O = no effect

Most extracts showed a depressor effect on toad heart, however extracts from *Boerhaavia coccinea*, *Byrsonima sericea*, *Curatella americana*, *Diodia barbeyana*, *Fagara rhoifolia*, *Lantana camara*, *Plumeria bracteata*, *Scoparia dulcis*, *Solanum paniculatum*, *Strychnos parvifolia*, *Tapirira guyanensis*, *Triplairis gardneriana*, *Willbrandia* sp. and *Zizyphus joazeiro* showed a stimulant effect.

Whereas extracts from *Cecropia carbonaria* (leaf) and *Simaruba versicolor* did exhibit a spasmolytic action upon guinea-pig ileum and rabbit duodenum, extracts from *Annona squamosa*, *Delonix regia* and *Fagara rhoifolia* (leaf) provoked a spasmogenic action on guinea-pig ileum and a spasmolytic one on rabbit duodenum. Extracts from *Pithecelobium multiflorum*, *Vitex gardneriana* and *Zizyphus joazeiro* promoted a spasmogenic action on both preparations.

Finally, oxytocic activity upon the rat uterus was shown by extracts from *Annona squamosa*, *Borreria verticilata*, *Byrsonima sericea*, *Cannabis sativa*, *Heliotropium indicum*, *Pithecelobium multiflorum* and *Vitex gardneriana*, whereas inhibition of the oxytocin-induced contractions was shown by extracts from *Coutarea hexandra*, *Simaruba versicolor* and *Tabebuia caraiba*.

DISCUSSION

Much of the literature on the plants examined deals with chemical aspects, few pharmacological results are reported.

Alpinia galanga and *A. officinarum* are official drugs used both as carminative and stimulant. *A. speciosa* is used as an hypotensive but we did not find this action, though there was a stimulant action, on smooth muscle.

Leaves of *Annona muricata* are used as a sedative and a hypnotic. Alkaloids are present in this genus and Burton (1963) studied the biosynthesis of annonaine, an alkaloid of *A. reticulata*.

Byrsonima crassifolia is used in folk medicine against fever, cold and snake-bite. Djerassi, Bowers & others (1956) and Matos, Alencar & others (1968) showed the presence of β-amirin in this species and in *B. sericea*.

Erythrina species have long been known to contain alkaloids with curare-like action. Wasicky & Unti (1952) found the seed extract of *E. crista galli* to possess molluscacidal activity against *Australorbis glabratus* (*sic*). Other species are used in folk medicine as hypnotics, anthelmintics and diuretics.

One of the plants most active on toad isolated heart was *Fagara rhoifolia*. Deulofeu (1946), Moisset de Espanés & Ortega (1946), Moisset de Espanés & Weksler (1946), Calderwood & Fish (1966), von Marquardt (1966) found tertiary and quaternary alkaloids in *Fagara* species. Kuck, Albanico & others (1967) examined the alkaloidal composition of seven *Fagara* species, among them *F. rhoifolia*. The alkaloids, mainly fagarine, reduce myocardial sensitivity and are used in auricular fibrillation, being in some ways superior to quinidine (Hocking, 1955).

The active principle of *Operculina macrocarpa*, the Brazilian jalap, is a purgative glycosidic resin. Some plants of the *Convolvulaceae* family have seeds containing alkaloid with psychotomimetic activity (Claus & Tyler, 1968). The chief active component is ergine, an amide of lysergic acid.

Of the *Cucurbitaceae*, *Luffa operculata* is used as a purgative and an abortifacient. Matos & Gottlieb (1967) identified Isocucurbitacin B, a cytotoxic substance, in the fruits of this plant. Djerassi & others (1956) found gypsogenin in the same plant.

The chemical constituents of *Crataeva roxhburghii* have been shown to be lupeol (Chakravarti, 1959) and an isothiocyanic glycoside, glucopparin (Kjaer & Thomson, 1962). Lupeol is also present in bark of *C. tapia* (Matos & others, 1968).

Cecropia species are cited as folk remedies for the treatment of oedema, asthma, liver diseases and as diuretics and cardioactive agents. According to Feng & others (1962), *C. peltata* presented toxicity to mice and promoted contraction of the guinea-pig ileum, but we didn't verify these effects in *C. carbonaria.*

Plants of the genus *Heliotropium* are rich in alkaloids (Petrova, Deniso & Men'Shikov, 1957; Brutko & Utkin, 1965), mainly heliotropine and casiocarpine. Some of these plants are toxic to mice (Avlyanova, Markmar & Umarov, 1965) but the extract of *H. indicum* was not toxic to mice and fish.

Lantana camara was screened by Hooper & Leonard (1965) whose results were similar to ours.

Matos (1960) found about 3% of total alkaloids in *Peschiera affinis.* Cava, Talapatra & others (1964), identified these as affinine and affinisine.

Pithecelobium saman contains pithecelobine, an alkaloid of unusual structure (Wiesner, 1960) having pharmacological properties (Leonard & Sherrat, 1961, 1967) similar to those of extracts of *P. multiflorum.* This species has a high alkaloid content (Matos & others, 1968).

Both *Solanum torum* and *S. incarnatum* contain steroidal glycosidic alkaloids (Ali, Khan & others, 1967; Fayez & Saleh, 1967).

Doepke (1962) found the alkaloid vitricine in *Vitex trifolia. V. agnus castus* has also been examined and the unsaponifiable part of the seed extract has a progesterone-like effect on rats (Belič, Bergont-Dolar & others, 1958). Ghatuvedi & Sing (1965) found extracts of this plant to have antirheumatic activity superior to salicylate but inferior to cortisone or butazolidine.

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The absorption, distribution and excretion of pentazocine in man after oral and intravenous administration

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Gas-liquid chromatography is a sensitive technique for the analysis of pentazocine in biological samples. Dose for dose, the concentration in blood and the urinary excretion rate of pentazocine are much lower after oral administration than after intravenous administration. The recovery of the unchanged drug in faeces is low whether it is given by mouth or intravenously.

The analgesic potency of pentazocine in man is much less when given orally than parenterally (Beaver, 1968). This may be due to a slow or incomplete absorption of the orally administered drug, or both. Evidence to support this view is lacking for pentazocine, but has been obtained in animals for the related analgesics morphine and racemorphan which behave similarly (Cochin, Haggart & others, 1954; Fisher & Long, 1953). A gas chromatographic procedure for the evaluation of pentazocine in biological samples has now been used to investigate the disposition of the orally and intravenously administered drug in man.

EXPERIMENTAL

Analytical methods

Apparatus. A Perkin-Elmer F11 chromatograph fitted with a flame ionization detector and coupled to a 0 to 5 mV Leeds and Northrup Speedomax G (model S) recorder was used. The chromatographic column was glass tubing, $\frac{1}{4}$ inch o.d., 2 m long, packed with 80–100 mesh Chromosorb G which was acid-washed, treated with chlorodimethylsilane and coated with 2·5% w/w SE30. The column was conditioned for 24 h under the operating conditions: injection port temperature about 250°, oven temperature 200°, nitrogen (carrier-gas) flow rate 60 ml/min. The inlet pressures of the flame gases were 25 lb/inch^2 for hydrogen and 35 lb/inch^2 for air.

Reagents. Ammonium hydroxide solution, 1·0N; hydrochloric acid 0·1N; internal marker solution, the equivalent of 5 μg base/ml of α-3-hydroxy-6-dimethylamino-4,4-diphenylheptane hydrochloride in distilled water; freshly distilled reagent-grade benzene‡; reagent-grade *n*-butanol.

Treatment of biological samples. Urine (5 ml), combined with internal marker solution (1 ml), was adjusted to pH 8·5–9·5 with ammonium hydroxide solution and extracted three times with benzene (2·5 ml). These extracts were combined in a test-tube, finely tapered at its base (Beckett, 1966).

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‡ Some benzene samples gave GLC peaks which interfered with the analysis of pentazocine at high instrument sensitivity; treatment with activated charcoal and redistillation was necessary.

Blood (2 ml), mixed with distilled water (3 ml) and internal marker (1 ml) was made alkaline with ammonium hydroxide (0·2 ml) and extracted three times with benzene (2·5 ml). The extracts were combined and extracted with hydrochloric acid (2·5 ml). The acidic phase was retained, washed with benzene (2 ml) made alkaline and extracted in the same way as urine. For larger blood samples, blood (15 ml) mixed with distilled water (10 ml) was made alkaline with ammonium hydroxide solution (1·5 ml), extracted three times with benzene (15 ml) and the combined benzene extract concentrated to approximately 7·5 ml and treated as above.

Faeces (whole sample) were homogenized in two volumes of hydrochloric acid. The homogenate was treated in the same way as blood.

Chromatographic analysis. The bulked benzene extracts were concentrated by evaporation at 90° on a water-bath. The concentrate was taken up into n-butanol (10 μl) just before complete evaporation of benzene and an aliquot (2–4 μl) was injected onto the gas chromatographic column with a 10 μl Hamilton syringe. The amount of pentazocine present in a sample was determined by measuring the peak-height ratio of pentazocine to internal marker and relating it to a previously constructed calibration graph. Retention times relative to solvent fronts were pentazocine 5·3 min, internal standard 3·7 min.

The specificity and reproducibility of the assay for pentazocine in biological samples and the recovery of drug by extraction. (i) Blood and urine samples from ten subjects and faecal samples from four subjects who had not received pentazocine were analysed. 16 replicate analyses were made of standard solutions of pentazocine in blood (0·2 μg/ml) and urine (1·0 μg/ml).

Distribution and excretion of pentazocine in man

General conditions of the trials. Except where stated, trials conditions were essentially those described by Beckett & Rowland (1965). Four healthy male volunteers, aged 23 to 42 years, participated. Urine was maintained acidic by orally administered ammonium chloride (Beckett & Tucker, 1966).

Intravenous administration. Pentazocine (24 mg base as lactate) in sterile aqueous solution (2 ml) was injected into a vein of the right forearm.

Oral administration. Pentazocine hydrochloride (100 mg) was taken in distilled water (50 ml).

Collection of biological samples. Control samples were collected before drug administration and analysed. After drug administration, blood samples (5 ml) were collected from a left forearm vein into heparinized vials at 10 min intervals for 2 h and larger samples (20 ml) were collected hourly for 6 h thereafter. Urine was collected half-hourly for the first 4 h, hourly for the next 4 h, 2-hourly for the next 6 h, then at 24 h and 4-hourly thereafter up to 32 h. Faeces were collected as passed, for up to 48 h after drug administration.

RESULTS

Analytical method

Specificity and reproducibility of the assay and recovery of drug. Pentazocine-free samples of blood, urine and faeces, when analysed at high instrument sensitivity, did not produce chromatographic peaks at, or close to, the retention time of the drug. The standard deviations of the assays of standard solutions of drug in blood and urine were 5 and 3% respectively. The average recoveries of drug by extraction from blood and urine were 98 and 93% respectively.

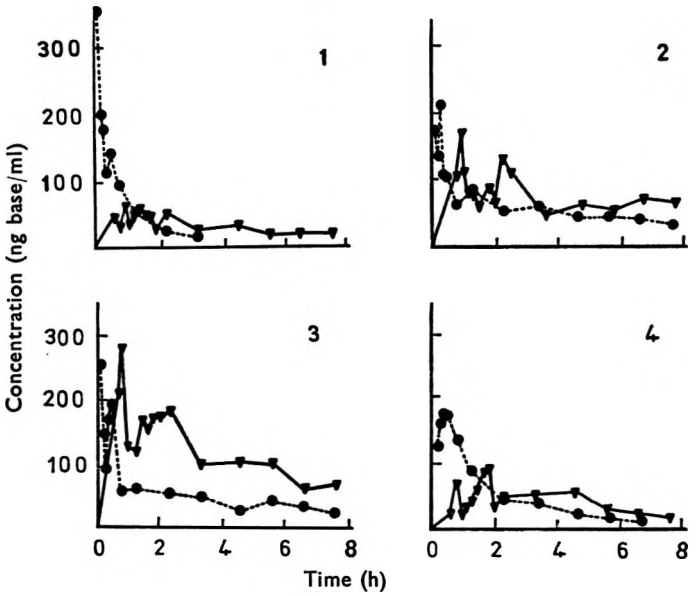

FIG. 1. Concentrations of pentazocine in blood after intravenous administration of 24 mg base as lactate (●– – – ●) and oral administration of 88·7 mg base as hydrochloride (▼——▼). Subjects 1–4.

Distribution and excretion of pentazocine in man

Concentration of pentazocine in blood. The values for successive blood samples after intravenous and oral administration of the drug are shown in Fig. 1.

Urinary and faecal excretion of pentazocine. Under conditions of maintained acidic urinary pH, the 32 h urinary recoveries of the dose as unchanged drug were 8 to 24% after intravenous administration and 3 to 15% after oral administration (Table 1). In each subject the percentage urinary recovery of dose was greater after

Table 1. *Urinary recoveries, urinary excretion half-lives and faecal recoveries of pentazocine after intravenous and oral administration to man under conditions of maintained acidic urinary pH*

Subject	Dose (mg base equivalent)	Route	Urinary pH	Urinary recovery of unchanged drug in 32 h (percentage dose)	Ratio of urinary recoveries (i.v./oral)	Urinary excretion half-life (h)	Faecal recovery of unchanged drug in 48 h (% dose)
1	24	i.v.	4·59–5·22	13·0	3·0	2·7	0·5
	88·7*	oral	4·70–6·25	4·4		2·5	0·4
2	24	i.v.	4·70–5·27	16·2	1·7	2·5	0·7
	88·7	oral	4·83–5·33	9·3		†	0·1
3	24	i.v.	4·67–5·31	24·0	1·6	6·0	0·7
	88·7	oral	4·67–5·41	15·1		5·5	1·5
4	24	i.v.	4·69–8·48†	8·4	2·8	†	0·1
	88·7	oral	4·87–5·41	3·0		†	0·5

* = 100 mg hydrochloride; † = non-linear terminal part of semi-log graph of excretion rate versus time.

† Less than 5·5 for most samples.

intravenous administration than after oral administration by a factor of 1·6 to 3·0. Excretion half-lives of pentazocine varied between subjects but not within subjects and appeared to be independent of the route of drug administration (Table 1). The 48 h recoveries of the dose as unchanged drug in the faeces were 0·1 to 2·0% and were apparently independent of the route of administration of drug (Table 1).

DISCUSSION

Effect of route of administration upon the entry of pentazocine into blood and excreta

Blood. Pentazocine given orally at a dose 3·7 times larger than that given intravenously produced blood concentrations only 1 to 2 times that following an intravenous dose (Fig. 1). Therefore, absorption of orally administered pentazocine is either slow or incomplete, or both. The concentrations of pentazocine in blood generally decline smoothly and exponentially with time after intravenous drug administration, but fluctuate with time after oral administration, the secondary and tertiary peaks indicating erratic absorption of drug.

Urine. The semi-logarithmic graphs of excretion rate versus time for intravenously and orally administered pentazocine were similar, despite the three-fold increase in oral dose, and almost linear after the initial absorption and distribution phases (Fig. 2), but some deviation can occur in the terminal part of the graph. These

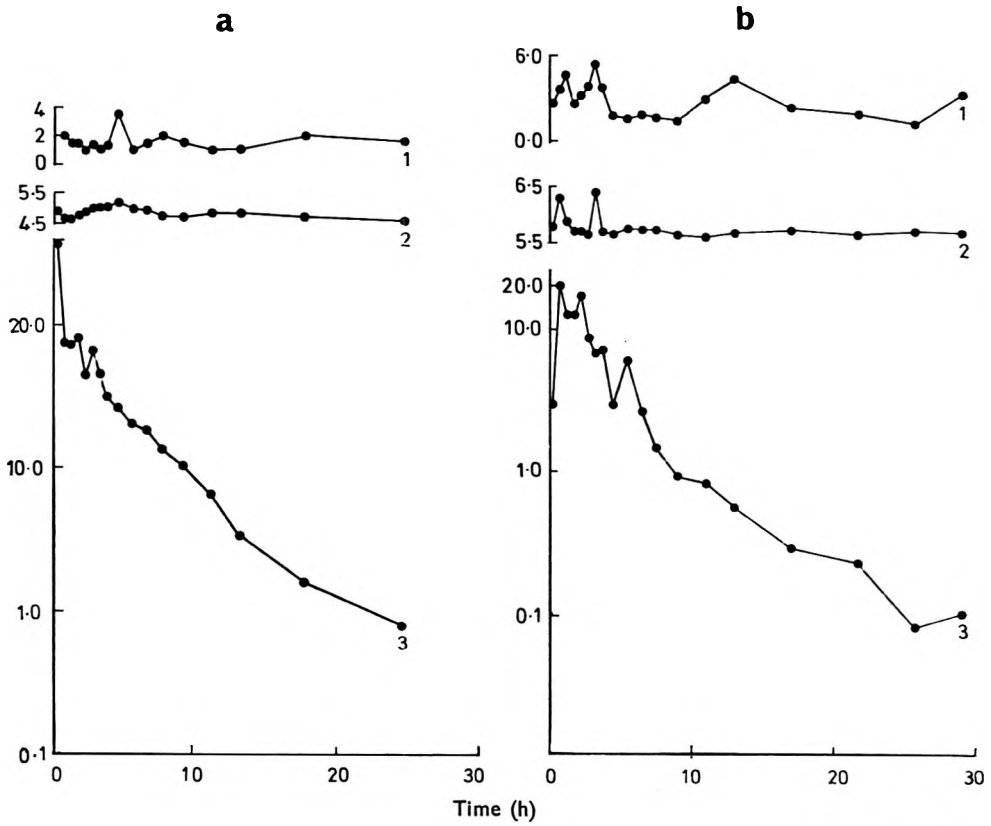

FIG. 2. Urinary excretion rate of pentazocine in μg/min (3), with corresponding urinary pH (2) and urine flow rate in ml/min (1) after (a) oral administration of 88·7 mg pentazocine base as hydrochloride; and (b), intravenous administration of 24 mg pentazocine as lacetate. Subject 1. Acid urine control.

observations confirm that pentazocine uptake from the gastrointestinal tract is impeded and erratic; slow absorption is unlikely to be the governing factor because the semilogarithmic plot of excretion rate versus time does not show the convex decreasing curvature considered indicative of this effect by Wagner (1963). Furthermore, if the urinary recovery of pentazocine under the described conditions is proportional to the amount of drug originally entering the body, the results (Fig. 3) indicate that about one to two thirds of the orally administered drug is absorbed to become distributed in the same way as an intravenous dose, i.e. there is incomplete absorption or organ clearance during absorption of 30 to 65% of the oral dose.

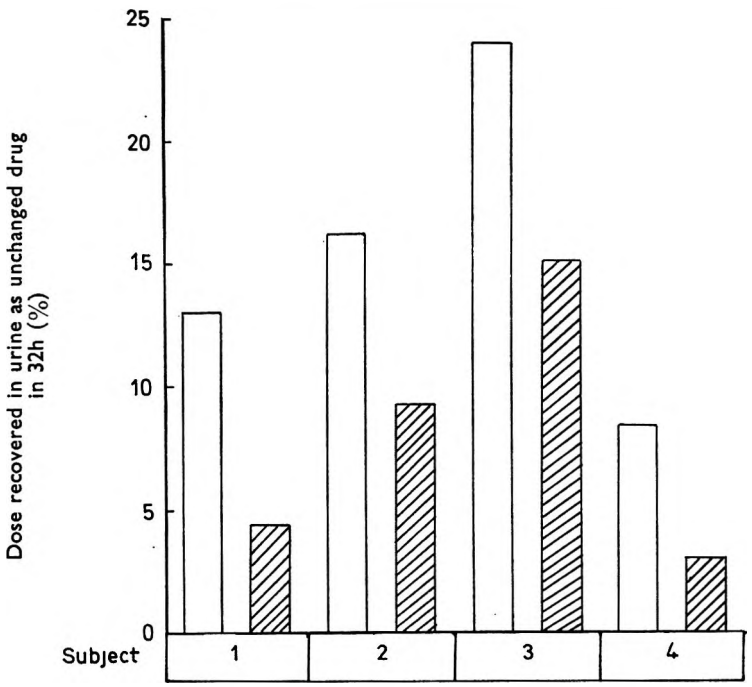

FIG. 3. Urinary recoveries of pentazocine after intravenous administration of 24 mg base as lactate (open columns), and oral administration of 88·7 mg base as hydrochloride (hatched columns) to man under conditions of maintained acidic urinary pH.

Faeces. The faecal recoveries of unchanged drug after administration by the intravenous and oral routes were low: 0·1 to 2·0% of the dose (Table 1). Therefore the "apparently" unabsorbed fraction of the oral dose is not accounted for in the faeces. These findings could be due to organ clearance of drug by metabolism during absorption, or biotransformation of the unabsorbed pentazocine by intestinal microorganisms. However, the second explanation is considered unlikely because metabolism by micro-organisms is generally confined to reductive and hydrolytic processes (Scheline, 1968) and pentazocine is metabolized by oxidation (Pittman, Rossi & others, 1969).

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LETTERS TO THE EDITOR

Influence of pretreatment with phenobarbitone on the ultrastructure of adrenergic nerve endings in guinea-pig seminal vesicles

Neither angiotensin nor tyramine contract the guinea-pig isolated seminal vesicle (Gascon & Walaszek, 1968; Gascon & Vaillancourt, 1969). After the addition of adrenaline, however, both compounds induce a contraction which exhibits tachyphylaxis. Furthermore, it has been shown (Gascon, Brodeur & Vaillancourt, 1969; Gascon & Brodeur, 1969) that chronic pretreatment of the guinea-pig with testosterone, phenobarbitone or other microsomal enzyme inducers, causes a marked increase in the reactivity of the isolated seminal vesicles to angiotensin and to tyramine.

We now report the influence of sub-acute pretreatment with phenobarbitone on the ultrastructure of the adrenergic nerve endings located in the guinea-pig seminal vesicle.

Twenty male guinea-pigs, 200–225 g, were divided at random into two groups. The animals of the first group were injected intraperitoneally with phenobarbitone (50 mg/kg daily) for 4 days, while those of the second group were given saline. 24 h after the last injection, the seminal vesicles were removed and immersed in a fixative mixture of glutaraldehyde-acrolein at 4°.

The middle portion of the organ was diced into blocks measuring a few millimetres; the blocks were kept for 1h in the fixative, and then post-fixed in osmium tetroxide for 3h (Caulfield, 1957), dehydrated in graded ethanols, and embedded in Epon 812 (Luft, 1961). Thin and ultrathin sections were cut on a Porter–Blum ultramicrotome, and triple-stained with lead, uranyl acetate, and lead again, for 5, 1 and 8 min, respectively (Sandborn, 1966). The ultrathin sections were examined in a Hitachi electron microscope HS–7S. The population of the small dense-core vesicles (40–60 nm), representing the neuro-transmitter noradrenaline (Wolfe, Potter & others, 1962; Bloom & Barrnett, 1966), was counted on 240 electron micrographs of nerve endings selected at random. In this experiment, 720 nerve endings were used, to ensure a total count of at least 3000 small dense-core vesicles.

There were slightly more than twice as many dense-core vesicles in the phenobarbitone pretreated than in the control nerve endings. The statistical analysis made on the absolute numbers of noradrenaline vesicles showed a highly significant difference ($P<0.001$) between the controls and the pretreated animals.

It has been reported (Gascon & Walaszek, 1968; Gascon & Vaillancourt, 1969) that angiotensin and tyramine induce a contraction of the guinea-pig isolated seminal vesicle by releasing endogenous catecholamine. Furthermore, it has been shown (Gascon & others, 1969; Gascon & Brodeur, 1969) that drugs such as testosterone and phenobarbitone, two microsomal enzyme inducers (Conney, 1967), modify the reactivity of the seminal vesicles to both agonists. This effect was attributed by Gascon and his co-workers to an enhanced synthesis of endogenous catecholamine. The increase in the population of the small dense-core vesicles reported here constitutes a strong argument in favour of this assumption. To our mind, this effect of phenobarbitone can be explained by an increase in the synthesis of new noradrenaline vesicles in the nerve endings located in the guinea-pig seminal vesicles. This hypothesis is supported by the studies of Mueller & Shideman (1968), who reported a phenobarbitone-induced decrease in the replenishing time of noradrenaline in the

hearts of animals previously treated with reserpine, and by the unpublished observations of Gascon & Cloutier, which showed a significant increase in the noradrenaline content of the seminal vesicles in animals so treated.

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Ulcerative colitis in rabbits fed degraded carrageenan

We recently described the occurrence of ulcerative colitis in guinea-pigs fed orally in their drinking water degraded and undegraded carrageenan derived from the red seaweed *Eucheuma spinosum* (Watt & Marcus, 1969). The lesions produced by degraded carrageenan were severe and involved the whole length of the colon.

We have found that degraded carrageenan derived from the same seaweed also causes severe damage to the colon of the rabbit. In this communication we report on the incidence of ulcerative lesions found in rabbits fed various concentrations of the degraded product.

Twenty male Californian rabbits of 2950 g average body weight were housed in separate cages and fed a standard cube diet (S.G.1). Three experimental groups, 5 rabbits in each group, received as drinking fluid 5, 1 and 0·1% respectively aqueous solutions of degraded carrageenan derived from *Eucheuma spinosum.** The solutions of degraded carrageenan were freshly prepared daily, stored at 4°, and supplied *ad lib* in drinking bottles which were cleaned each day. The volume of fluid consumed per animal per day was measured throughout the 6 to 12 week period of the experiment. Control animals received water *ad lib* but without added carrageenan. At weekly intervals, the animals were weighed and their faeces examined for occult blood using the Haematest method. At the end of the experiment, the animals were killed with pentobarbitone. At post-mortem examination, the colon was removed, emptied of faeces and examined for the presence of ulcerative lesions.

Animals fed degraded carrageenan at the 5% concentration in their drinking water received on average a daily dose of 1·4 g/kg weight over a 6 week period. Diarrhoea associated with visible and occult blood in the faeces developed by the end of 7 days and persisted. The animals rapidly lost weight, the average loss at the end of the

experiment being 976 g. All animals in this group showed severe ulceration of the colon.

Animals fed degraded carrageenan at the 1% concentration received on average a daily dose of 0·8 g/kg weight over a 7 week period. Only 1 animal developed diarrhoea; occult blood was present in the faeces in all of the animals after 2 weeks. The average weight loss in the group was 190 g. All of the animals showed ulceration of the colon of moderate severity.

Animals fed degraded carrageenan at the 0·1% concentration received on average a daily dose of 0·07 g/kg over a 12 week period. Diarrhoea did not occur but occult blood in the faeces was demonstrable in 3 of the animals by the end of 10 weeks. One animal lost 30 g in weight; the remainder gained weight, the average weight gain in the group being 238 g. Multiple ulcers in the colon were found in 3 of the 5 rabbits.

Control animals drank on average 370 ml water per day over a 12 week period. There was no diarrhoea or occult blood in the faeces at any time. The average gain was 1218 g. No ulcerative lesions were found in the colon in any of the control animals.

The results indicate that degraded carrageenan at doses of 0·07 g/kg per day will produce ulcerative lesions in the colon in 3 out of 5 rabbits when given orally in the drinking fluid over a period of 3 months. It is possible that an even higher incidence of lesions may have been found had the feeding experiment been prolonged.

In this country, degraded carrageenan derived from *Eucheuma spinosum* has been used only in clinical trials in the treatment of chronic peptic ulcer (Evans, Lowell & Thomas, 1965). On the continent, however, the product in tablet form is widely used to reduce peptic activity and gastric acidity in gastro-duodenal disorders (Bonfils, 1968). The maximal dose recommended per day is about the same (0·07 g/kg) as produces ulcerative lesions in the colon of the rabbit.

In the light of these findings, together with our results in three other animal species (Marcus & Watt, 1969), it seems that the continued use of degraded carrageenan as a drug in the treatment of peptic ulcer may not be without risk.

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* Carrageenan degraded by mild acid hydrolysis and retaining about 29% sulphate was kindly supplied by Laboratoires Glaxo, Paris.

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Nicotinic activity of choline

In recent years a number of reports from this laboratory noted that cholinergic effects in plant extracts were due to choline (Das & Sanyal, 1964; Gambhir, Sanyal & others, 1966; Sanyal, Dasgupta & others, 1966; Bhattacharya, Lal & others, 1969). The presence of choline was characterized by physico-chemical studies like paper chromatography, determination of melting point and mixed melting point. We found that the presence of choline in a plant extract could be predicted with certainty if a comparison of the muscarinic and nicotinic effects of the extract, or the base isolated from it, was compared with acetylcholine. A definite and differential relation was found to exist between the muscarinic and nicotinic actions of acetylcholine and choline used as their chloride salts. Our findings are summarized in Table 1.

Table 1. *Comparison of the muscarinic and nicotinic activities of acetylcholine chloride and choline chloride*

Experimental Parameter	Acetylcholine	Choline chloride	Relative Potency
Muscarinic action			
(a) Depressor response in anaesthetized dog (pentobarbitone sodium 35mg/kg, i.p.)	1 μg/kg, i.v.	3·5 mg/kg, i.v.	1 : 3500
(b) Spasm of isolated ileum of rabbit	0·005 μg/ml	25 μg/ml	1 : 5000
Nicotinic action			
(a) Pressor response in atropinized (1 mg/kg, i.v.) anaesthetized dog	100 μg/kg, i.v.	2 mg/kg, i.v.	1 : 200
(b) Spasm of isolated rectus abdominis muscle of frog	1 μg/ml	0·2 mg/ml	1 : 200

Doses represent a mean of 20 experiments

The results clearly indicate that though choline chloride is approximately 3000 to 5000 times less potent than acetylcholine chloride in terms of muscarinic activity, its nicotinic action is only about 200 times weaker. Thus choline has a relatively more potent nicotinic action. This is equally true at both ganglionic and neuromuscular junctional sites. We feel that the nicotinic activity of choline should be better appreciated. A quantitative assessment of the muscarinic and nicotinic actions of a plant product showing cholinergic activity with standard acetylcholine can give the investigator a good lead as to whether he is dealing with choline or not.

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Duration of effect of atropine sulphate against the toxicity of oxotremorine in mice

The central actions of oxotremorine may be blocked with atropine sulphate but not with methylatropine (Lévy & Michel-Ber, 1966). Mice excrete 38% of a dose of [^{3}H]oxotremorine within 2 h of administration (Hammer, Karlén & others, 1968). Within 48 h of injection of [^{14}C]atropine sulphate, however, mice excrete 80–90% of the ^{14}C (Albanus, Sundwall & others, 1968). This information indicates the duration of residence of the radioactive atom in the body, but not that of the biologically active compound. To assess the duration of effect of atropine sulphate in mice, therefore, the duration of its protection against the lethal effects of oxotremorine, a compound which stimulates peripheral and central receptors sensitive to atropine blockade, was examined.

Female albino mice, CF No. 1 strain, 18–24 g, bred in these laboratories were divided into groups of 10. All drugs were dissolved in physiological saline and administered in a dose volume of 1 ml/100 g body weight. A dose-mortality curve to intravenously injected oxotremorine sesquifumarate (Aldrich Chemical Co., Inc.) was determined from which the LD99 (maximum lethal dose) was estimated to be 30 mg/kg. Further groups of mice were then injected intraperitoneally with graded doses of atropine sulphate, B.P. and challenged at measured time intervals thereafter with the intravenous LD99 of oxotremorine. The number of mice surviving at each dose level of atropine at the different time intervals was recorded after 24 h (Table 1), and was found to be inversely related to the time interval between atropine pretreatment and the oxotremorine challenge at each dose level of atropine. Plotting % survivors on the ordinate against time of pretreatment on the abscissa produces a series of parallel lines, which are displaced to the right as the dose of atropine is increased. The duration of protection of 50% of a group of mice (PT50) was calculated and found to be a sigmoid function of the $\log_{10}$ dose of atropine. Thus, the *duration* of protection of mice against the lethal effects of oxotremorine by atropine is a function of the dose.

Table 1. *Duration of protection of mice by atropine sulphate against the lethal effects of oxotremorine sesquifumarate* (30 mg/kg i.v.)

Atropine sulphate i.p. (mg/kg)	% surviving at different time intervals after treatment (min)										PT50 (min)
	30	60	90	120	150	180	210	240	270	300	
1·25	90	60	0	—	—	—	—	—	—	—	59·4
2·50	100	70	10	0	—	—	—	—	—	—	67·5
5·0	100	100	60	20	0	—	—	—	—	—	97·5
10·0	100	100	—	—	—	90	60	0	—	—	210·0
20·0	100	100	100	90	—	90	90	100	30	10	252·6

Kords, Lüllmann & others (1968) reported the degree of protection of mice by atropine sulphate injected 15 min before acutely toxic doses of di-isopropylfluorophosphonate to be unrelated to the dose of the cholinoceptor blocking agent. The results of the experiments now reported show that, even at the lowest doses examined, atropine sulphate may confer 100% protection against the LD99 of oxotremorine, providing the time interval between its injection and pretreatment with atropine is sufficiently short.

I wish to thank Mr. G. R. Ellender for his skilled assistance.

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When is a drug inactive? Concerning the uricosuric activity of some anti-inflammatory drugs

Many acidic drugs are extensively bound to plasma proteins and it has been generally accepted that the bound fraction has no pharmacological activity (Goldstein, 1949; Brodie, 1965). This concept certainly needs modifying in the light of recent reports that one drug may displace another from a common binding site on the albumin molecule; for example, salicylate and phenylbutazone can displace thyroxine and certain sulphonamides, sulphonylureas and oral anticoagulants (Solomon & Schrogie, 1967; Meyer & Guttman, 1968).

Such an interaction between two drugs *in vivo* can be formulated as follows:

Species A + Albumin–Species B combination $\rightleftharpoons$ Species B + Albumin–Species A.

Thus one drug (A) may have an "adjuvant," or potentiating, action on the pharmacological activity of another drug (B) by either increasing its effective concentration (as unbound drug B) or otherwise making it more readily available to its responsive receptors. Drug A would, however, acquire and demonstrate this adjuvant activity only when it was itself bound to the albumin. We wish to extend and further illuminate this concept that when albumin-bound, a drug entity may "acquire" pharmacological activity, which need not necessarily be that of enhancing the activity of another drug or shortening its biological half-life.

Experiments in our clinic showed that the oral ingestion of certain drugs by healthy adult volunteers significantly lowered, in a reversible manner, the capacity of the plasma proteins to bind uric acid (Bluestone, Kippen & Klinenberg, 1969). Most of this urate-binding capacity (*ca* 70%) is associated with the albumin fraction. When the urate-binding capacity of human albumin preparations both in the presence and absence of added drugs *in vitro*, was measured (Table 1) it was found that a number of anti-inflammatory acids effectively inhibited urate binding to human albumin *in vitro*, and that in man, aspirin and phenylbutazone could probably displace uric acid from its albumin binding site(s) *in vivo*.

These observations suggested that phenylbutazone and salicylates are useful uricosuric drugs because they displace some of the albumin bound urate and so augment its renal clearance. When albumin bound, phenylbutazone and salicylate may well be inactive as analgesics, antipyretics or anti-inflammatory agents; but at the same time they could also have a uricosuric effect, quite apart from any other uricosuric activities they might have when not bound to albumin, e.g., acting directly on the kidneys. In fact it may now be helpful to subdivide drugs, currently classed as "uricosurics" according to whether they (*a*) can inhibit tubular reabsorption of urate, (*b*) can displace urate from its binding sites in plasma or other tissues, or

Table 1. *Displacement of urate from its albumin-binding site(s) by some anti-inflammatory anions and probenecid*

Drug	ED50 *in vitro** (mM)	Efficacy *in vivo*†	
		Dose (g/day)	Response
Aspirin	0·3	3·0	+++
Sodium salicylate ..	0·1		N.D.
Phenylbutazone ..	0·15	0·4	++
Sulphinpyrazone ..	0·25	0·4	—
Indomethacin	0·3	0·15	—
Mefenamic acid ..	0·1	1·5	+
Probenecid	0·3	1·5–3·0	++

N.D. = not determined.

* Concentration of drug inhibiting urate binding by 50% in PIPES buffer, pH 7·35 containing 0·75 mM crystalline human serum albumin (Pentex Inc., Kankakee, Illinois) and 0·9 mM uric acid, determined by equilibrium dialysis at 4° (Klinenberg & Kippen, 1970). Saturated solutions of colchicine and allopurinol were almost devoid of activity.

† Ability to reduce plasma urate-binding capacity (determined *in vitro*) after ingestion of drug by at least 3 normal healthy adults for at least 2 days; see Bluestone & others (1969). Key: +++ = over 70% inhibition, ++ = over 40% inhibition, + = over 20% inhibition.

(*c*) facilitate urate excretion by yet some other means—while recognizing that a given uricosuric drug may enhance urate excretion by more than one of these actions at the same time.

To summarize: we would like to re-emphasize that whilst a drug is retained within the body, it should *always* be considered potentially active, even when bound to "silent-receptors" such as the albumin drug-binding sites. A drug might still cause some unexpected and apparently unrelated pharmacological (or pathological) effects by displacing certain hormones, other drugs or even normal metabolites (e.g., bilirubin, uric acid) from their usual binding sites, including the appropriate catabolic enzymes.

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Effect of antidepressant drugs on accumulation and disappearance of monoamines formed *in vivo* from labelled precursors in mouse brain

Tricyclic antidepressant drugs prevent the uptake of monoamines into noradrenaline and 5-hydroxytryptamine (5-HT) neurons (Dengler & Titus, 1961; Glowinski & Axelrod, 1964; Carlsson, Fuxe & others, 1966; Carlsson, Corrodi & others, 1969a, b), an effect which does not lead to changes in the levels of monoamines in tissues (Sulser, Watts & Brodie, 1962; Nybäck, Borzecki & Sedvall, 1968; Carlsson, & others, 1969a, b).

We have recently described the use of labelled tryptophan and tyrosine for the study of drug influences on monoamine metabolism in mouse brain (Schubert, Nybäck & Sedvall, 1970; Nybäck & Sedvall, 1970), methods that have the advantage that endogenous levels of brain amines are left unchanged. We now report the effect of some tricyclic antidepressants on the accumulation and disappearance of labelled 5-HT (^{3}H-5-HT), dopamine and noradrenaline formed in mouse brain *in vivo* after the administration of [^{3}H]tryptophan or [^{14}C]tyrosine intravenously.

5-HT *metabolism.* After an intravenous injection of [^{3}H]tryptophan to mice (male, NMRI, 18–22 g) the 5-HT store in brain is maximally labelled within 30 min (Schubert & others, 1970). Between 1–3 h after administration of the labelled precursor, ^{3}H-5-HT disappears from the brain at a rate which appears to be exponential and which is not increased by treatment with the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (Schubert & others, 1970). Thus the disappearance of labelled 5-HT during the mentioned time interval is determined predominantly by the turnover rate of the amine.

Imipramine, desipramine, amitriptyline and nortriptyline (25 mg/kg) were administered intraperitoneally 1 h after the intravenous injection of [^{3}H]tryptophan (50 μCi/animal, 1·3 Ci/mmol). Groups of animals were killed 1 and 3 h after the precursor administration and the contents in brain of labelled tryptophan and 5-HT were determined as previously described (Schubert & others, 1970).

The dimethylated agents, amitriptyline and imipramine, retarded the rate of disappearance of ^{3}H-5-HT whereas the monomethylated derivatives had no significant ($P<0·05$) effect (Table 1).

When the labelled precursor was administered by constant rate intravenous infusion for 20 min, the rate of ^{3}H-5-HT accumulation in brain was reduced by pretreatment of the animals with imipramine and chlorimipramine (Table 2).

Table 1. *Effect of some antidepressant drugs on the disappearance of* ^{3}H-5-HT *formed from* ^{3}H-*tryptophan in mouse brain.* Saline or drugs (25 mg/kg) were administered 1 h after i.v. injection of [^{3}H]tryptophan (50 μCi). Animals were killed 3 h after [^{3}H]trytophan administration. Figures represent mean value ± s.e. from 7 animals

Treatment	Time h	Total radioactivity counts/min × 10^3 g^{-1}	[^{3}H]Tryptophan counts/min × 10^3 g^{-1}	^{3}H-5-HT counts/min × 10^3 g^{-1}
Saline	1	84 ± 5·3	9·4 ± 0·5	1·72 ± 0·09
Saline	3	84 ± 5·8	3·4 ± 0·4	0·73 ± 0·04
Imipramine ..	3	75 ± 6·1	4·1 ± 0·5	0·99 ± 0·07*
Desipramine ..	3	72 ± 3·8	3·1 ± 0·3	0·85 ± 0·04
Amitriptyline ..	3	79 ± 6·0	3·5 ± 0·3	1·05 ± 0·08*
Nortriptyline ..	3	74 ± 2·9	2·6 ± 0·4	0·74 ± 0·06

* Differs from 3 h saline group ($P<0·01$).

Desipramine, nortriptyline and amitriptyline did not significantly affect the ^{3}H-5-HT accumulation in brain. A reduction of the ^{3}H-5-HT accumulation by amitriptyline could, however, be masked by an increased precursor supply as indicated by the increased content of labelled tryptophan in the brain (Table 2).

Table 2. *Effect of some antidepressant drugs on the accumulation of* ^{3}H-5-HT *formed from* [^{3}H]*tryptophan in mouse brain.* [^{3}H]Tryptophan (40 μCi) was infused i.v. for 20 min starting 40 min after injection of saline or drugs (25 mg/kg). Animals were killed immediately after the infusion. Figures represent mean value ± s.e. from 6–8 animals

Treatment	Total radioactivity Counts/min × 10^3 g^{-1}	[^{3}H]Tryptophan Counts/min × 10^3 g^{-1}	^{3}H-5-HT Counts/min × 10^3 g^{-1}
Saline	113 ± 6·8	62 ± 2·5	1·39 ± 0·09
Imipramine	109 ± 7·0	69 ± 4·6	0·85 ± 0·05*
Desipramine ..	114 ± 4·0	63 ± 3·9	1·15 ± 0·07
Amitriptyline ..	138 ± 3·6	85 ± 4·7*	1·18 ± 0·08
Nortriptyline ..	117 ± 8·1	75 ± 6·1	1·21 ± 0·05
Chlorimipramine ..	115 ± 5·3	60 ± 5·9	0·77 ± 0·10*

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

Table 3. *Effect of some antidepressant drugs on the disappearance of catecholamines formed from* [^{14}C]*tyrosine in mouse brain.* Saline or drugs were administered 2 h (10 mg/kg) and 3, 4, 5 and 6 h (5 mg/kg) after injection of [^{14}C]tyrosine (10 μCi). Animals were killed 7 h after [^{14}C]tyrosine administration. Figures represent mean value ± s.e. from 4–6 animals

Treatment	Time h	Endogenous tyrosine μg/g	Tyrosine sp. activity counts/min μg^{-1}	[^{14}C]Dopamine counts/min g^{-1}	[^{14}C]Noradrenaline counts/min g^{-1}
—	2	9 ± 0·8	344 ± 28	757 ± 53	413 ± 17
Saline	7	10 ± 1·1	144 ± 24	216 ± 12	165 ± 20
Imipramine ..	7	9 ± 0·7	123 ± 21	227 ± 27	175 ± 12
Desipramine ..	7	9 ± 0·5	126 ± 13	217 ± 8	164 ± 12
Amitriptyline ..	7	9 ± 1·2	142 ± 7	265 ± 29	153 ± 13
Nortriptyline ..	7	8 ± 0·9	159 ± 25	250 ± 14	160 ± 10

Table 4. *Effect of some antidepressant drugs on the accumulation of catecholamines formed from* [^{14}C]*tyrosine in mouse brain.* [^{14}C]Tyrosine (7 μCi) was infused i.v. for 20 min starting 40 min after injection of saline or drugs (10 mg/kg). Animals were killed immediately after the infusion. Figures represent mean value ± s.e. from 4–6 animals

Treatment	Endogenous tyrosine μg/g	Tyrosine sp. activity counts/min × 10^3 μg^{-1}	[^{14}C]Dopamine counts/min g^{-1}	[^{14}C]Noradrenaline counts/min g^{-1}
Saline	14 ± 0·7	4·2 ± 0·29	980 ± 88	270 ± 20
Imipramine	12 ± 1·4	4·9 ± 0·62	1014 ± 88	237 ± 26
Desipramine	12 ± 1·1	5·1 ± 0·69	820 ± 100	160 ± 21†
Amitriptyline	11 ± 1·1	5·9 ± 0·32*	1060 ± 95	329 ± 24
Nortriptyline	12 ± 0·9	4·8 ± 0·51	873 ± 71	178 ± 16†

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

Catecholamine metabolism. After an intravenous injection of [^{14}C]tyrosine, labelled dopamine and noradrenaline accumulate in mouse brain during the first 30 min (Nybäck & others, 1968). Between 2 and 7 h after the precursor administration, the amines disappear from brain at a rate which seems to be exponential and which is not increased by synthesis inhibition with α-methyltyrosine (Nybäck & Sedvall, 1970). Thus the disappearance of labelled amines during the mentioned time interval is determined predominantly by the turnover rates of the amines.

The antidepressant drugs were administered intraperitoneally 2 h (10 mg/kg) and 3, 4, 5 and 6 h (5 mg/kg) after the intravenous injection of [^{14}C]tyrosine (10 μCi/animal, 355 mCi/mmol). Groups of animals were killed 2 and 7 h after the precursor administration and the contents in brain of endogenous tyrosine and labelled tyrosine, dopamine and noradrenaline were determined as previously described (Nybäck & Sedvall, 1968, 1970).

None of the antidepressants altered the rate of disappearance of labelled dopamine or noradrenaline from brain (Table 3).

When [^{14}C]tyrosine was administered by constant rate intravenous infusion for 20 min the accumulation of [^{14}C]noradrenaline was significantly reduced by pretreatment with desipramine and nortriptyline but not by their dimethylated derivatives (Table 4). None of the drugs caused any significant change in the accumulation of [^{14}C]dopamine. Amitriptyline increased the specific activity of tyrosine in brain ($P<0{\cdot}02$).

The present results demonstrate significant effects of dimethylated antidepressants on brain 5-HT metabolism but not on noradrenaline or dopamine metabolism. In contrast, the monomethylated derivatives had a significant effect on the metabolism of brain noradrenaline but not on that of dopamine or 5-HT. These findings are consistent with results obtained by other investigators using different methods (Carlsson & others, 1969a, b; Corrodi & Fuxe, 1969; Schildkraut, Schanberg & others, 1969; Ross & Renyi, 1969). The reduced amine accumulation and disappearance, found in the present study, indicate that dimethylated and monomethylated antidepressants decelerate synthesis and turnover of the transmitter in serotoninergic and noradrenergic neurons respectively. Such an effect could be mediated by the inhibition of amine re-uptake, if this leads to an increased receptor stimulation which by a negative feed-back mechanism inhibits nerve impulse activity in the presynaptic neuron.

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Inability of dextran to release kinin in rats

Ankier & Starr (1967) showed the hypotensive response to intravenous dextran in rats to be probably mediated by histamine and 5-hydroxytryptamine. Lecomte & Damas (1968) now suggest that plasma kinins may have an accessory role in this anaphylactoid reaction. I now report that dextran does not release kinin in rats.

Male Wistar albino rats, 150–175 g (Wellcome Laboratories, Beckenham, and Agricultural Research Council, Compton) were tested for reactivity to dextran on three occasions (250 mg/kg, i.p., *M* 110,000), and those which showed peripheral oedema (reactors) were separated from non-reactors. Reactor rats were anaesthetized with pentobarbitone (40 mg/kg, i.p.) and injected with heparin (50 units/kg, i.v.). Blood pressure was recorded from the right common carotid artery with a Condon mercury manometer, and drugs injected into the right femoral vein.

Dextran (250 mg/kg) elicited a fall in blood pressure in reactor rats which lasted 40–60 min, after an initial delay in onset of 2–5 min. A similar response was produced by ellagic acid (5 mg/kg), an activator of Hageman factor and plasma pre-kallikrein (Margolis, 1958; Ratnoff & Crum, 1964), but with a quicker onset (1–2 min) and a shorter duration (10–15 min). Tachyphylaxis developed to repeated injections of ellagic acid, indicating a progressive consumption of the substrate for plasma kallikrein in the blood (substrate 1—Jacobsen, 1966). However, the administration of dextran to kininogen-depleted rats still caused a typical fall in blood pressure. Pretreating rats with Soya Bean Trypsin Inhibitor (SBT1, 100 mg/kg) reduced the hypotensive activity of ellagic acid by about 64%, whereas this concentration did not affect the action of dextran (Table 1). From these observations it seems unlikely that dextran activates plasma kinin-forming enzymes to a significant extent, since inhibition of plasma kallikrein with SBT1, or reducing the level of its substrate in the blood, did not modify the course of the reaction to dextran. On the other hand, both of these procedures attenuated the hypotensive action of ellagic acid, a compound which is known to activate plasma kallikrein. Another possibility also investigated was that dextran in some way liberated glandular

Table 1. *Effects on the blood pressure responses to dextran in reactor rats of soya bean trypsin inhibitor (SBT1, 100 mg/kg, i.v.) and ellagic acid, and of Trasylol (100 000 units/kg, i.v.) and pancreatic kallikrein*

Drug	dose (mg/kg)	Inhibitor	% fall in blood pressure (± s.e.) Untreated	After inhibition
Dextran	250	SBTI	58·8 ± 6·9	54·3 ± 2·3
Ellagic acid	5	SBTI	49·5 ± 4·8	17·8 ± 1·2*
Dextran	250	Trasylol	65·1 ± 9·2	58·2 ± 5·0
Pancreatic kallikrein	150	Trasylol	27·4 ± 2·4	13·4 ± 2·8*

* Inhibition = 64% ($P < 0.001$).
* Inhibition = 51% ($P < 0.005$).

kallikreins from tissue stores, which then released kinins from a separate plasma substrate (substrate 2—Jacobsen, 1966). Intravenous injections of pancreatic kallikrein (150 units/kg; Glumorin, Bayer) produced rapidly-developing and short-lasting reductions in blood pressure, but as many as eight consecutive doses usually had to be given to obtain a pronounced tachyphylaxis to this enzyme. Even when rats had been depleted of substrate 2—kininogen in this way, the degree of hypotension obtained with dextran in such animals lay within the normal range. In addition, rats previously treated with Trasylol (100,000 units/kg, i.v.; Bayer), an inhibitor of glandular kallikreins (Werle & Maier, 1952), showed a diminished reactivity to pancreatic kallikrein (51% inhibition) but not to dextran (Table 1). Thus, it is considered that glandular kallikreins do not play an active part in the blood pressure response to dextran in the rat.

Non-reactor rats have been found to be resistant to the blood pressure effects of injected dextran (Ankier & Starr, 1967), and yet in the present experiments they were found to be just as sensitive as reactors to intravenously-administered ellagic acid, pancreatic kallikrein and synthetic bradykinin (2·5–10 μg/kg; BRS 640, Sandoz). These results confirm earlier findings of the qualitative and quantitative similarities of the plasma kinin systems of the two kinds of rat (Ankier & Starr, 1967). In view of these similarities, it is difficult to account for reactivity on the basis of a selective liberation of plasma kinins, or non-reactivity as being a defect in the mechanism for kinin formation and release.

In conclusion, the above experiments show that neither the selective depletion of the substrates for kinin-forming enzymes in the blood, nor the selective inhibition of the enzymes themselves, modify significantly the vasodepressor property of dextran in the albino rat. The relative unimportance of plasma kinins in the early stages of the anaphylactoid syndrome is further emphasized by the apparent normality of the plasma kinin system in rats which failed to react to dextran. This is not to say that kinins may not be involved in the later process of oedema formation, when it is highly probable that a localized liberation of kinins, occurs secondary to the release of other chemical mediators, such as histamine and 5-hydroxytryptamine (Ankier & Starr, 1967; Greeff, 1968).

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Hydroxytryptamine turnover decreased by the antidepressant drug chlorimipramine

The antidepressant action of imipramine and related drugs has been ascribed largely to a blockade of one of the amine transporting mechanisms of the central monoamine containing neurons. This uptake mechanism, the membrane pump, is probably located at the level of the nerve cell membrane, and may form a major means of inactivating the neurotransmitter released into the synaptic area after nerve stimulation. The uptake mechanism and its blockade by imipramine-like agents have been studied extensively in noradrenaline-containing neurons, both centrally and peripherally (Andén, Carlsson & Häggendal, 1969). Recent experiments have revealed a similar uptake-concentration mechanism also in central 5-hydroxytryptamine (5-HT)-containing neurons. This membrane pump, too, is blocked by imipramine and, still more effectively, by chlorimipramine (Andén & others, 1969; Carlsson, Corrodi & others, 1969a; Carlsson, Jonason & others, 1969b). Imipramine has also been shown to slow the turnover rate of brain 5-HT (Corrodi & Fuxe, 1968; Schildkraut, Schanberg & others, 1969). Blockade of re-uptake may cause an increased amount of 5-HT to reach its receptors, or may cause the released 5-HT to remain near the receptors longer. It has been suggested that antidepressants cause an increase in receptor stimulation. Negative feedback mechanisms might then cause the impulse frequency of the 5-HT nerve to decrease, and thus lower the rate of 5-HT turnover. Since these antidepressant drugs may act partly by virtue of their influence on 5-HT neurons, it should be worthwhile to examine these alterations further.

Two methods have been used to examine the effects of antidepressants on 5-HT turnover. In the first, a tryptophan hydroxylase inhibitor was used to block 5-HT synthesis (Corrodi & Fuxe, 1968). Pretreatment with imipramine partly prevented the resulting 5-HT depletion. Therefore, it appeared that the rate of 5-HT breakdown or leakage was decreased. However, the correct turnover rates are difficult to calculate from such experiments, since decreasing the 5-HT concentration by synthesis inhibition may by itself slow the turnover. The second method involved intracerebral injection of labelled 5-HT and noting its disappearance (Meek, 1968; Schildkraut & others, 1969). In two separate investigations, imipramine slowed the disappearance of intracerebrally injected ^{14}C-5-HT. However, the injected amine may label only a small part of the endogenous pool, which might lead to erroneous conclusions.

A third approach takes advantage of the fact that there is only one detectable metabolite of 5-HT in brain, 5-hydroxyindoleacetic acid (5-HIAA). If no 5-HIAA can leave the brain, and no exogenous 5-HIAA can enter the brain, then the rate of 5-HT breakdown can be measured by determining the rate of accumulation of 5-HIAA. Probenecid appears to block the active efflux of 5-HIAA from brain (Sharman, 1966; Neff, Tozer & Brodie, 1967; Werdinius, 1967a; Diaz, Ngai & Costa, 1968). Neff & others (1967) have suggested that this blockade is complete, and that neither probenecid, nor the increased 5-HIAA level alters 5-HT metabolism. We have used this method to estimate the changes in 5-HT breakdown produced by chlorimipramine.

Male Sprague–Dawley rats, 150–200 g were injected intraperitoneally with chlorimipramine (15 mg/kg). Fifteen min later, they and control rats received probenecid (200 mg/kg, i.p.). At intervals, the animals were decapitated, brains from three rats pooled and analysed for 5-HIAA (Werdinius, 1967b). Four or five determinations were made at each interval for each of the two treatments.

Fig. 1 shows the effect of chlorimipramine on the accumulation of 5-HIAA. In animals treated only with probenecid, brain 5-HIAA levels increased linearly (0 22 μg/g h^{-1}) for 2 h. The animals pretreated with chlorimipramine accumulated

5-HIAA more slowly (0·068 μg/g h^{-1}). The difference in slopes (Davies, 1949) was statistically significant ($P<0{\cdot}005$).

The rate of accumulation of 5-HIAA after probenecid was lower than that reported by Diaz & others (1968) (0·29 μg/g h^{-1}) or by Neff & others (1967) (0·40 μg/g h^{-1}). A difference between strains of rats is one possible cause for this discrepancy. Brodie & others (1966) examined catecholamine turnover rates in rats from two different sources, and found one twice as fast as the other.

If the accumulation of 5-HIAA is to be a measure of 5-HT turnover, the 5-HT concentration must remain constant. In a separate series of experiments in this laboratory, chlorimipramine (15 or 25 mg/kg, i.p.) did not alter brain 5-HT levels.

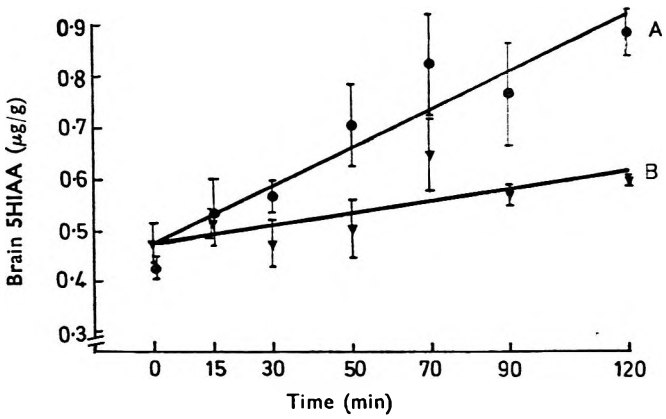

FIG. 1. Accumulation of 5-HIAA in rat brain after probenecid blockade of 5-HIAA efflux. Probenecid (200 mg/kg, i.p.) was injected at time zero to untreated controls, (A), or to animals pretreated with chlorimipramine (15 mg/kg, i.p.), 15 min earlier (B). The values represent mean ± s.e. of 4 or 5 determinations, each comprising 3 pooled whole brains.

From the present experiments, it seems likely that the 5-HT turnover rate of rats treated with chlorimipramine was only 30% of that of control animals. The cause of this effect on turnover is uncertain. Two possibilities are that chlorimipramine facilitated diffusion of 5-HIAA or antagonized the blockade of active efflux by probenecid. However, since two other independent methods show that imipramine-like drugs alter 5-HT turnover, it seems more likely that the change in 5-HIAA accumulation was a result of reduced the 5-HT oxidation. Possible explanations may be that 5-HT was prevented from reaching monoamine oxidase, or that rate of release of 5-HT declined as a consequence of some negative feed-back mechanism.

In addition to turnover studies, a variety of other techniques show that antidepressant drugs affect 5-HT in the central nervous system. For example, imipramine and chlorimipramine prevent depletion of brain 5-HT by the displacing agent, 4-methyl-α-ethyl-*m*-tyramine (Carlsson & others, 1969), probably by blocking the neuronal uptake of the displacing amine. Imipramine, but not desmethylimipramine, blocks uptake of intracerebrally injected 5-HT. There is also histochemical and *in vitro* evidence (Carlsson & others, 1969) to suggest that extraneuronal 5-HT concentration rises as a result of imipramine or chlorimipramine treatment.

Our findings lend further support to the possibility that antidepressant drugs may act, at least partly, by altering some effect of 5-HT in brain.

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Prevention of experimental gastric ulcer in rats by a substance which increases biosynthesis of acid mucopolysaccharides

Biosynthesis of acid mucopolysaccharides, essential components of the connective tissue, has been much studied. The simplest approach is to investigate the incorporation of $^{35}SO_4$ into cartilage. The uptake of $^{35}SO_4$ is inhibited by steroid and non-steroid anti-inflammatory agents, both *in vitro* and *in vivo* in a dose-response relation (Bollet, 1961; Whitehouse & Boström, 1962; Szigeti, Ezer & others, 1965; Ezer & Boström, 1968).

ε-*p*-Chlorocarbobenzoxy-L-lysin-OMe-HCl (KL–11), increased the incorporation of $^{35}SO_4$ into the cartilage of the rat *in vivo* (Szporny, Ezer & others, 1969) and also prevented the inhibition of uptake of ^{35}S caused by prednisolone.

More and more importance is now attached to acid mucopolysaccharides that are present in large amounts in the gastric mucous membrane. Denko (1958) has shown that administration of hydrocortisone to hypophysectomized rats reduced the incorporation of ^{35}S into the tissues of the stomach. Kent & Allen (1966) have found that the $^{35}SO_4$ and glucose-U-^{14}C uptake by the gastric mucosa can be inhibited by sodium salicylate. It now seems equally certain that a significant inhibition of the synthesis of acid mucopolysaccharides can be achieved in the gastric mucous membrane by anti-inflammatory drugs. Perrey (1968) has described a parallel between the erosion of the gastric mucous membrane and the inhibition of glucosamine-6-phosphate synthesis by salicylate treatment. Since great importance is attached to the mucin content of the gastric mucous membrane in protecting the gastric wall against gastric juices, particularly hydrochloric acid, it seems that the damaging effect of anti-inflammatory substances in inhibiting the synthesis of mucopolysaccharides arises in this way.

Table 1. *Incorporation of* $^{35}SO_4$ *in stomach* in vivo *after* KL–11, *sodium salicylate or phenylbutazone*

Treatment Drugs	Doses mg/kg, i.p.	Specific activity of stomach counts/min of each 100 mg of dry tissue Control (no treatment)	Treatment	Change %
KL–11	25	3520 ± 97*	12300 ± 472	+270
KL–11	50	3970 ± 103	18600 ± 1060	+370
Sodium salicylate	500	4530 ± 154	3120 ± 124	− 31
KL–11 + sodium salicylate	50 + 500	4215 ± 137	5700 ± 226	+ 36
Phenylbutazone	150	3870 ± 124	2830 ± 157	+ 27
KL—11 + phenylbutazone	50 + 150	3210 ± 116	10980 ± 484	+340
Fasting for 48 h	—	3725 ± 153	1880 ± 83	− 50

* = s.e. of 10 animals.

Table 2. *The inhibiting effect of KL–11 on the development of Shay ulcers*

Doses mg/kg s.c.	Inhibition of ulcers (%) relative to control value By number	Scoring rate
15	75·3	58·5
25	82·0	69·5
50	94·0	75·0

We have now examined the way in which KL–11 influences the sulphate metabolism of the gastric wall. Male Wistar rats, 100–120 g, were given simultaneously a single dose of KL–11 and $^{35}SO_4$, 50 μCi/100 g, per animal. Radioactivity of the gastric wall was measured 20 h after treatment. It is evident (Table 1) that KL–11 is dose-related in increasing the incorporation of $^{35}SO_4$. Also the incorporation of $^{35}SO_4$ into the gastric mucous can be decreased significantly by sodium salicylate or phenylbutazone. After fasting, $^{35}SO_4$-uptake by the stomach was also diminished. The simultaneous use of KL–11 totally compensated for the effect of the anti-inflammatory drugs and when given simultaneously with phenylbutazone, the two drugs caused a significant increase of sulphate uptake at one control value.

We also investigated the effect of KL–11 on experimental gastric ulcers. Male rats, 100–120 g, were fasted for 64 h and then 6 h after ligating the pylorus, the gastric wall was investigated (Shay, Komarov & others, 1945). The ulcers were evaluated according to Shay & others (1945) and Bonta (1961). KL–11 was administered at the same time that the pylorus was ligated and it was found to inhibit the development of ulcers significantly (Table 2).

According to our hypothesis KL–11 inhibits the onset of experimental gastric ulcer by increasing the synthesis of mucopolysaccharide of the gastric mucous membrane. Thus there seems to be a possibility for developing substances with a new mechanism of action which may play a role in preventing the damaging effect of anti-inflammatory substances exerted on the stomach, and eventually also in the therapy of ulcer.

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Differentiation of β-adrenoreceptors by the use of blocking agents

There is evidence that the β-adrenoreceptor population is comprised of at least two types designated as β-1 and β-2 (Lands, Arnold & others, 1967; Lands, Luduena & Buzzo, 1967). This sub-division was proposed to account for the differing structural requirements of catecholethanolamines for initiating β-sympathomimetic actions in different organs. The sub-division so proposed has been further supported by the recent discovery of β-agonists, such as salbutamol, with selective β-2 actions (Cullum, Farmer & others, 1969).

If β-adrenoreceptors differ significantly in their structural requirements for agonists then it is reasonable to suppose that such receptors could have different structural requirements for antagonists. Thus experiments have been made to measure quantitatively the β-adrenoreceptor blocking action (by use of pA_2 measurements) of two compounds, propranolol and ICI 50 172 (against isoprenaline) at typical β-1 and β-2 type receptors. Previous workers have shown that tissues with similar receptors can be expected to give the same pA_2 with a given antagonist (Arunlakshana & Schild, 1959). ICI 50 172 was chosen in addition to propranolol because some selectivity of blocking action for this compound has been described (Dunlop & Shanks, 1968).

Table 1. pA_2 *values for propranolol and ICI* 50 172 *on isolated tissues of the guinea-pig, rabbit and rat. Isoprenaline was used as an agonist*

Species	Preparation	Receptor type	Propranolol	ICI 50 172
Guinea-pig	Atria-force rate	β-1	8·8	7·3
			8·6	7·3
Rabbit	Ileum		8·7	5·9
Guinea-pig	Trachea	β-2	8·7	5·4
"	Vas deferens		8·9*	6·8*
Rat	Uterus		8·5	5·0

* pA_2 value determined in the presence of 2 μg/ml cocaine.

Table 1 gives pA_2 values for propranolol and ICI 50 172 on isolated tissues of the guinea-pig, rabbit and rat. The pA_2 measurements were made by the method of Arunlakshana & Schild (1959) and each value was the mean of three determinations. The β-adrenoreceptor of the guinea-pig vas deferens although not previously classified, is, on the basis of work done in this laboratory, a β-2 type. Propranolol gave similar pA_2 values at both β-1 and β-2 type receptors and thus showed no selectivity in its blocking action. However, ICI 50 172 had a selective blocking action but did not always differentiate between β-1 and β-2 types since the compound showed highest activity on heart (β-1) and vas deferens (β-2) and much lower activity on ileum (β-1) and trachea (β-2).

These results agree with the concept of Lands and others that there are different β-receptor mechanisms in different tissues but are difficult to reconcile with their simple two receptor hypothesis.

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Antagonistic effects of dopa and propranolol on brain glycogen

We have reported previously that propranolol raises the glycogen content of the brain (Estler & Ammon, 1966, 1967), but we could not decide whether this effect was attributable to the anti-adrenergic effect of propranolol, which, by lowering the cyclic 3′,5′-AMP content of the brain, should inhibit glycogen breakdown and favour glycogen synthesis, or to the central depressant properties of propranolol, described by Leszkovsky & Tardos (1965), Murmann, Almirante & Saccani-Guelfi (1966) and Estler & Ammon (1969), that could likewise depress glycogenolysis. A decision seemed to be possible, however, on the assumption that the blockade of the adrenergic receptors is competitive (Wang, 1967) and should be overcome by large amounts of catecholamines. Experiments were therefore made on mice treated simultaneously with propranolol, and dopa which was chosen since unlike catecholamines, it crosses the blood brain barrier and penetrates into the brain where it is converted to dopamine and noradrenaline (Hornykiewicz, 1966; Marley, 1966). Dopa should thus antagonize the anti-adrenergic effects of propranolol if given in sufficient amounts.

Female NMRI-mice, kept at 25°, were treated with (±)-propranolol (5 μg/g, i.p.) or with (±)-dopa (300 μg/g, i.v.) or with both. 30 or 60 min later they were killed by immersion in liquid air. The brains were removed while still frozen and glycogen was measured (Kemp & Kits van Heijningen, 1954). Motility was measured in circular activity cages (Estler & Ammon, 1969).

As in our previous experiments (Estler & Ammon, 1966, 1967), propranolol did not significantly affect the spontaneous motor activity of single mice, but the glycogen content of the brain was increased (Table 1). The behavioural effect of dopa may range from central stimulation to central depression, depending on the species, dose and experimental condition (Boissier & Simon, 1966; Hornykiewicz, 1966). In our experiments 300 μg/g of dopa much reduced the motor activity of mice and temporarily lowered the glycogen content of the brain, a decrease probably attributable to the glycogenolytic action of catecholamines derived from dopa. After 1 h, brain glycogen concentrations returned to normal. In this way the effects of dopa are in contrast to those of other central depressants, which raise the glycogen content of the brain (Ammon, Estler & Heim, 1965), but resemble those of ethanol

Table 1. *Effects of propranolol and dopa on motor activity and cerebral glycogen in mice*

Treatment	Spontaneous motor activity (Impulses/30 min) min after treatment 0–30	30–60	Cerebral glycogen (μmol glucose equivalents/g) min after treatment 0	30	60
Controls	138 ±11 (34)	73 ±11 (34)	6·36 ±0·20 (14)	—	—
(±)-Propranolol .. .. 5 μg/g, i.p.	134 ±17 (24)	60 ±11 (24)	—	6·85* ±0·23 (13)	7·60* ±0·28 (12)
(±)-Dopa .. .. .. 200 μg/g, i.v.	44* ± 6 (35)	25* ± 4 (35)	—	5·81* ±0·20 (12)	6·30 ±0·21 (12)
(±)-Propranolol .. .. 5 μg/g, i.p.+ .. .. (±)-Dopa .. .. 200 μg/g, i.v.	46* ± 7 (35)	23* ± 4 (35)	—	6·24† ±0·20 (14)	6·98*† ±0·20 (12)

Mean values and standard errors of the means (s.e.), number of animals in parentheses.
* Value significantly different from the control, $P \leqslant 0{\cdot}05$.
† Significantly different from mice treated with propranolol alone, $P \leqslant 0{\cdot}05$.

which depresses CNS function and transitorily decreases the cerebral glycogen content by releasing catecholamines from their stores (Ammon & others, 1965; Estler & Ammon, 1965). When given simultaneously with propranolol, dopa prevented the increase of the glycogen content within the first 30 min after its injection. Later, the glycogen rose at the same rate as in the animals treated with propranolol alone.

Our experiments show that the β-receptor blocking agent propranolol and the sympathomimetic drug dopa have similar effects on brain function but antagonistic effects on brain glycogen. It seems reasonable to assume that the glycogen metabolism of the brain is controlled by adrenergic mechanisms and that the increase of brain glycogen produced by propranolol is the result of the β-receptor blocking rather than the central depressant properties of propranolol.

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The effect of change of solvent on the critical micelle concentration of a non-ionic surfactant

Little attention has been devoted to the physical properties of non-ionic surfactants in either non-aqueous solvents, or in mixed solvents in which water is a component. In mixed solvent systems the critical micelle concentration (CMC), of the surfactant usually varies with solvent composition. It would be useful to be able to predict, accurately, the CMC of a surfactant in any solvent, or mixture of solvents, from a knowledge of some property of the solute or solvent. This is unlikely. However, for certain systems, it should be possible to derive an approximate relation between the CMC of a surfactant and some property of a mixed solvent, such that, if two different solvent systems have the same value for this property, the CMC of a surfactant might be expected to be the same in both solvents.

To illustrate this, the CMC values of a non-ionic surfactant, dodecyl hexaoxyethylene glycol monoether ($C_{12}E_6$), have been measured, or interpolated from plots of CMC against temperature, at 25°, in water, formamide and mixtures of water and formamide, water and *N*-methylformamide (NMF) and water and dimethylformamide (DMF). The CMC values, which were measured by the drop-volume surface tension method, are shown in Table 1. All the CMC values increase with increase in the concentrations of amides in the solvents. In relating these increases in CMC to changes in solvent composition, two factors are considered.

The first factor is the effect of solvent dielectric constant on CMC values. For example, the dielectric constants of water and formamide at 25° are 78·5 and 109·5 respectively. As the amounts of formamide in the solvent mixtures increase the dielectric constant will also increase towards that of formamide. Conversely, in H_2O–DMF mixtures the dielectric constant will decrease as the concentration of DMF increases, as the dielectric constant of DMF at 25° is 36·7. Table 1 shows that it would be possible for $C_{12}E_6$ to have the same CMC in H_2O–formamide and H_2O–DMF mixtures that would have widely differing dielectric constants. The same conclusion is reached if micellization in H_2O–NMF mixtures is compared with micellization in the H_2O–formamide and H_2O–DMF mixtures. Therefore, for the systems discussed, solvent dielectric constants are of little importance in predicting CMC values and this agrees with previous findings (Schick & Gilbert, 1965).

The second factor which may be considered is the effect of solvent solubility parameters (δ) on CMC values (Eqn 1) (Hildebrand & Scott, 1964a),

$$\delta = \left(\frac{\Delta H - RT}{V}\right)^{\frac{1}{2}} \qquad (1)$$

where ΔH = heat of vapourization of liquid, V = molar volume of liquid. The solubility parameter of a liquid measures the amount of energy required to vapourize one cc of liquid, and is an indication of the forces holding the molecules of liquid together. In the derivation of δ no assumptions are made about polarity, association,

Table 1. *Critical micelle concentrations of $C_{12}E_6$ in various solvents (all* w/w*) at* 25°

Solvent	CMC (mol fraction)	Solvent	CMC (mol fraction)	Solvent	CMC (mol fraction)
H_2O	$1{\cdot}58 \times 10^{-6}$	25% NMF	$1{\cdot}81 \times 10^{-5}$	30% DMF	$2{\cdot}63 \times 10^{-5}$
25% Formamide ..	$1{\cdot}07 \times 10^{-5}$	50% NMF	$2{\cdot}29 \times 10^{-4}$	44% DMF	$1{\cdot}03 \times 10^{-4}$
42% Formamide ..	$2{\cdot}49 \times 10^{-5}$	75% NMF	$1{\cdot}72 \times 10^{-3}$	59% DMF	$3{\cdot}54 \times 10^{-4}$
55% Formamide ..	$3{\cdot}83 \times 10^{-5}$			65% DMF	$6{\cdot}87 \times 10^{-4}$
90% Formamide ..	$3{\cdot}46 \times 10^{-4}$			75% DMF	$2{\cdot}20 \times 10^{-3}$
Formamide ..	$1{\cdot}24 \times 10^{-3}$				

aggregation or solvation. This may seem to preclude the estimation of solubility parameters of polar liquids, such as are used here. However, if the parameters are used with discretion they provide useful, if approximate, guides to the solvent properties of polar liquids. The apparent solubility parameters of water, formamide, NMF and DMF are 23·4, 19·4, 16·1 and 12·2 respectively (Walker, 1952; Bauder & Gunthard, 1958; Hildebrand & Scott, 1964b). Several equations have been used to calculate solubility parameters of mixed solvents (Burrell, 1955), none of which is completely satisfactory. In the present work, since CMC values are quoted as mol fractions, the apparent solubility parameters of the solvents are calculated in terms of mol fractions of solvent constituents (Eqn 2)

$$\delta_{12} = \delta_1 X_1 + \delta_2 X_2 \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where δ_1, δ_2 and X_1, X_2 are solubility parameters and mol fractions of constituents 1 and 2 respectively. A plot of the logarithms of CMC values against apparent solubility parameters of the solvents is shown in Fig. 1. In systems in which the CMC values are low and the aggregation numbers are high, the logs of the CMC values are directly proportional to the free energies of micellization (ΔG°). Since no aggregation numbers are available, for the present systems, it can be said only that there may be a simple relation between the logs of the CMC and ΔG° values, and

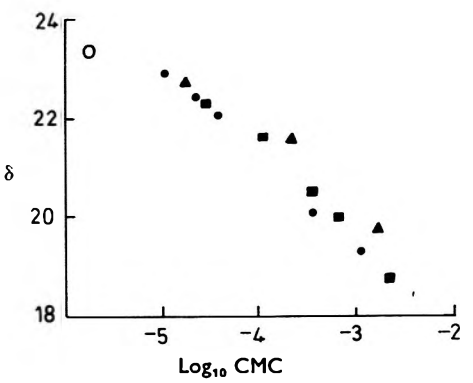

FIG. 1. Plot of logs of CMC values of $C_{12}E_6$ in various solvents against apparent solubility parameters (δ) of solvents.
(○ H_2O. ● H_2O–formamide. ▲ H_2O–NMF. ■ H_2O–DMF).

hence a relation between free energy of micellization of a surfactant and the solubility parameter of the solvent. It can be seen that, allowing for errors in the values of the solubility parameters, it would be possible to estimate the CMC of $C_{12}E_6$ in any of the systems studied from a knowledge of the solubility parameter of the solvent.

Too few CMC measurements, in too few solvent systems are presented to allow the assumption that a general relation exists between CMC and the solubility parameter of any solvent. Also, such factors as interactions between surfactant and amides and water and amides are ignored in the present treatment and may be important (Emmerson & Holtzer, 1967).

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The estimation of aqueous solutions of ω-diazoacetophenones by polarography

The involvement of the diazo group in the inhibition of purine synthesis has been demonstrated by Levenberg, Melnick & Buchanan (1957), thus making the study of this group important in biological systems. We have investigated the effects of a group of ω-diazoacetophenones, on several biological systems both *in vivo* and *in vitro*. During the course of this work we have found that existing methods of estimating these compounds in aqueous buffers, and in solutions containing organic material, are inadequate.

ω-Diazoacetophenones have characteristic absorption bands in the ultraviolet region of the spectrum (Leveson & Thomas, 1966), and this property has been used to determine the concentration of these compounds. For instance, Aziz & Tillet (1968) studied the rate of hydrolysis of diazoketones by following spectrophotometrically the decrease in their characteristic absorbances. In solutions containing protein this method is unsatisfactory, due to interference in the absorption at about 280 nm.

We have found a convenient way of assaying ω-diazoacetophenones is by polarography. The DC polarographic behaviour of ω-diazoacetophenone has been

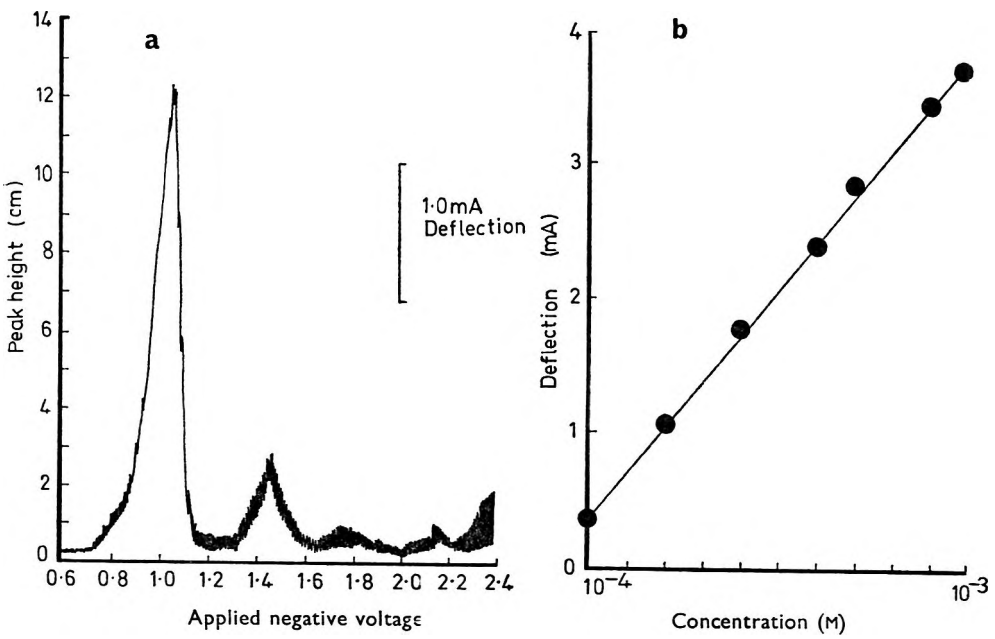

FIG. 1. a. AC polarogram of 10^{-3}M α-diazoacetophenone in 0·2M phosphate buffer at pH 6·7. The height of the primary peak is dependent on the concentration of the ω-diazoacetophenone. Substituted ω-diazoacetophenones (10^{-4}–10^{-3}M) give similar traces under the same conditions.

b. The calibration curve of ω-diazoacetophenone in 0·2M phosphate buffer, pH 6·7, at 25°, using AC polarography.

reported (Foffani, Salvagnini & Pecile, 1959; Coombs & Leveson, 1964), and the behaviour of some nuclear substituted diazoacetophenones in aqueous buffer solutions has been described (Bailes, 1968). The polarograms typically show three waves, the first of which (a six electron wave) is concentration dependent, and can be used for quantitative analysis. In our work we have used AC rather than conventional polarography. This modification gives a trace that is suitable for assay purposes (Fig. 1a). Characteristically it shows a primary well defined peak, the height of which is concentration dependent, followed by two smaller peaks, which do not interfere with the measurement of the primary peak.

For ω-diazoacetophenones which are sufficiently soluble, it is convenient for routine assay purposes to construct a calibration curve as shown in Fig. 1b. For insoluble ω-diazoacetophenones we used the calibration curve for the unsubstituted compound in the assay. This is accurate only if the diffusion current constants are the same for the unsubstituted and substituted compounds. Values of the diffusion current constant ($\mu A\ mg^{-2/3}\ s^{1/2}\ mM^{-1}$), I, for five relatively soluble ω-diazoacetophenones ($x\text{-}C_6H_4\cdot CO\cdot CHN_2$) in 0·2M phosphate buffer, pH 6·7 at 25°, calculated from the results of Bailes (1968) are:

X =	H	*m*-Me	*p*-Me	*p*-OMe	*m*-Cl	*m*-F
	10·10	9·67	9·67	9·67	8·70	10·13

Although the effect of nuclear substitution on I is generally slight, it must be appreciated that there is the possibility of introducing a large error by using the technique; for example, the I value for *m*-chloro-ω-diazoacetophenone.

The polarograph used was the Cambridge general purpose model, fitted with a Univector unit. By this means a small alternating potential was superimposed upon the DC applied to the cell. The polarogram was obtained by plotting the alternating component of the current against the electrode potential. All determinations were carried out in Cambridge Instrument Co. cells. The solutions were de-oxygenated for 10 min with nitrogen before readings were taken.

Solutions of the ω-diazoacetophenones were prepared in 0·2M phosphate buffer at the pH required. Saturated solutions were prepared by shaking excess of the solid in about 10 ml of phosphate buffer at 25° for 24 h. For estimation, these solutions were transferred, with undissolved solid, to the polarographic cell, maintained at 25° by means of a water jacket.

It seems likely that polarography will prove a satisfactory method for assaying other diazo-ketones, and using AC polarography, traces in a convenient form for assay purposes can be obtained.

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Testing of acid-citrate-dextrose anticoagulant solution for absence of pyrogens

Acid-citrate-dextrose (A.C.D.) anticoagulant solution is an ingredient of Whole Human Blood B.P. and, as such, may be administered intravenously in large quantities during blood transfusion. This suggests that a test for pyrogenicity would be a worthwhile precaution.

The British Pharmacopoeia does not require that this solution be tested for pyrogens, while the United States Pharmacopoeia describes a dilution method of testing and the Swiss Pharmacopoeia refers to the use of calcium gluconate to eliminate the risk of tetany in rabbits. A.C.D. solution has a pH of 5 and, as well as the discomfort this causes, tetany-like spasms have been noticed in rabbits after intravenous injection of 10 ml/kg.

To overcome these effects the use of a basic calcium salt that would neutralize the acidity and supply calcium ions has been investigated. Calcium carbonate was chosen and when added to A.C.D. solution (0·5 g/120 ml) containing sodium acid citrate (2 g) the salt raised the pH to about 6·5 and on intravenous injection the solution no longer produced signs of discomfort or tetany at 10 ml/kg in the rabbit.

The salt was sterilized by heating 0·5 g (B.P. grade) quantities, weighed into Universal bottles capped with aluminium foil, and heated at 250° for 1 h in a hot-air oven. This treatment was also expected to destroy any pyrogen. Immediately before making a pyrogen test, 0·5 g of the sterilized calcium carbonate was added to each 120 ml quantity of A.C.D. solution and the containers were placed in a water-bath at 40°. The slight excess of calcium salt settled out quickly leaving a clear supernatant available for injection.

To confirm that this was a reliable test of pyrogens in A.C.D. solution, 0·036 μg of Organon "E Pyrogen" in solution was added to 360 ml of a sterile anticoagulant solution which had been prepared using full precautions against the development of pyrogens. Sterile calcium carbonate (1·5 g) was then added and a pyrogen test was made on the supernatant fluid. Three rabbits were each given 10 ml/kg and this produced a total rise in temperature of 2·4° which was within the range expected from this pyrogen in doses of 0·001 μg/kg rabbit.

The same amount of the "E Pyrogen" solution was added to 1·5 g of calcium carbonate. This was immediately freeze-dried to eliminate the water and the dry calcium carbonate with pyrogen was heated in a hot-air oven at 250° for 1 h. The heated material was then added to 360 ml of the above batch of sterile A.C.D. anticoagulant solution and a pyrogen test was made as before. The total rise in temperature for three rabbits was 0·4° which indicated that the pyrogen had been destroyed.

Thus a satisfactory pyrogen test can be made on A.C.D. anticoagulant solution, using 10 ml/kg, if 0·5 g of calcium carbonate powder, previously heated at 250° for 1 h is added for each 2 g of sodium acid citrate in the solution. The addition of heated calcium carbonate does not add pyrogen nor does it inhibit the expected response to a known dose of a standard pyrogen. The test has the advantage that no dilution is required as in the U.S.P. method and that unlike calcium gluconate, calcium carbonate can be rendered pyrogen-free by heating.

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Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, U.K. GEORGE M. TODD

November 24, 1969

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Further particulars are available from the Secretary-General, Association of Commonwealth Universities, (Appts.), 36 Gordon Square, London, W.C.1, or from the Registrar of the University.

Applications close in New Zealand and London on **28 February 1970**

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