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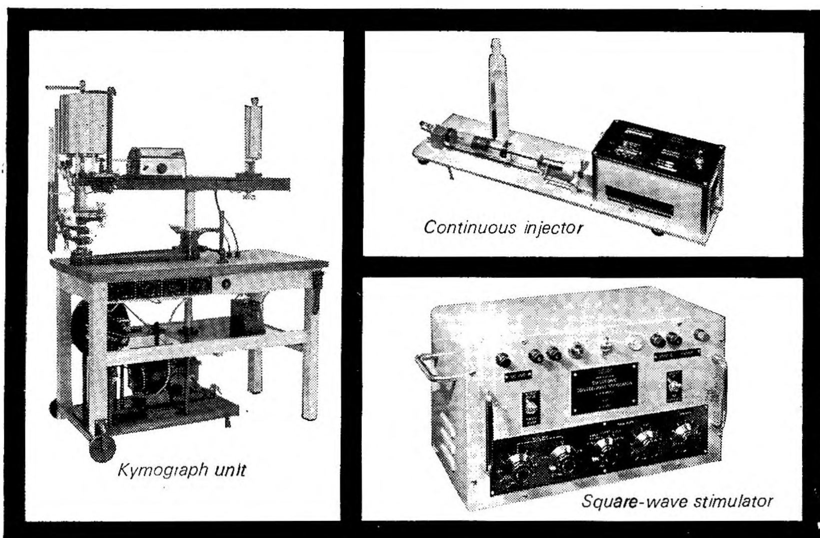
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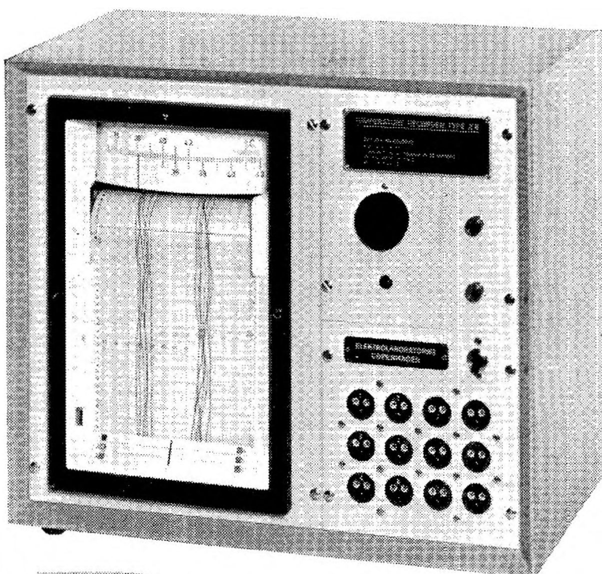

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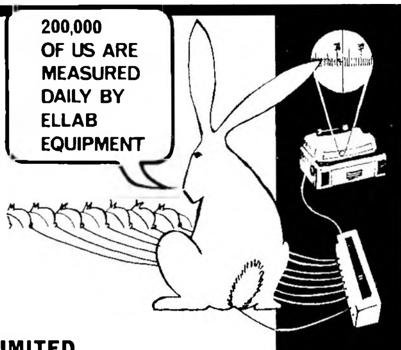

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The effects of drug-induced adrenocortical deficiency and of mineralocorticoid drugs on anaphylaxis in the guinea-pig

R. HICKS

Administration of adrenocortical inhibitors [metyrapone, amphenone-B, or 1,1-dichloro-2,2-di(*p*-chlorophenyl)ethane (DDD)] decreased anaphylactic preconvulsion times in guinea-pigs. Simultaneous corticotrophin treatment potentiated the effect of metyrapone. Metyrapone induced adrenocortical deficiency, and changed the character of corticosteroid secretion from predominantly glucocorticoid to mineralocorticoid. Mineralocorticoid treatment decreased preconvulsion times. Mepyramine prevented the effect of mineralocorticoids on anaphylaxis but did not modify the effect of metyrapone which probably resulted from glucocorticoid lack, but mineralocorticoid secretion may have contributed. Mineralocorticoids probably potentiated the histaminic component of anaphylaxis.

IN 1922, Kepinow claimed that an almost complete bilateral adrenalectomy increased the severity of guinea-pig anaphylaxis, while Banting & Gairns (1926) reported that adrenalectomy decreased the resistance of guinea-pigs to administered histamine. Gross & Haefeli (1952) used adrenalectomized animals maintained with deoxycortone, and demonstrated increased severity of anaphylactic reactions. On the other hand, Benaim, Feinberg & Sternberger (1955) were unable to show any increase in the severity of reactions to aerosols of either antigen or histamine, in hypersensitive guinea-pigs which were stated to have been completely, bilaterally, adrenalectomized. Moreover, Takaishi (1935) and Bongiovanni (1957) have claimed that adrenalectomy decreased the severity of anaphylactic and histamine shocks in this species.

A possible reason for the contradiction may lie in the technical difficulties of performing complete bilateral adrenalectomy in the guinea-pig. It is now possible to induce adrenocortical deficiency by administration of drugs which inhibit corticosteroid secretion. I have used some compounds of this nature to investigate the effects of adrenocortical deficiency on anaphylaxis in the guinea-pig.

Experimental

MATERIALS AND METHODS

Virgin female albino guinea-pigs of the Dunkin Hartley strain, weighing 300–500 g, and maintained on unrestricted supplies of water, and diet S.G.I. (Oxoid) supplemented by hay and green vegetables, were used. They were housed in well ventilated conditions, with the temperature regulated at 65° F.

Active hypersensitivity was induced by single intraperitoneal injections of crystalline egg albumen (50 mg/kg) in aqueous solution. Reactions were induced 28 days after sensitization.

Antigen-aerosol induced anaphylaxis. Sensitized animals were placed in

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a closed chamber and exposed to an aerosol of 5% egg albumen solution. The aerosol was produced by a "Wright" nebulizer using compressed air at a pressure of 10 lb/inch^2. The severity of the resultant anaphylactic reaction was assessed according to the method of Herxheimer (1952). Evaluation was in terms of the "preconvulsion time," i.e. the time of exposure required to elicit consistently recognizable signs of the onset of respiratory distress, such that the immediate removal of the animal from the aerosol would avert convulsions and death. Unless otherwise stated all experiments were made using groups of 5 animals, and results were taken as the mean values of individual preconvulsion times. Effects of drug treatment were evaluated by comparison with control groups treated with the inert vehicle used in drug administration.

Experimental and results

EFFECTS OF REPEATED DAILY ADMINISTRATION OF DRUGS INHIBITING ADRENOCORTICAL SECRETIONS

Daily intramuscular doses of either 1,1-dichloro-2,2-di(*p*-chlorophenyl)-ethane (DDD) (50 mg/kg), amphenone-B (200 mg/kg) or metyrapone (200 mg/kg) were administered to groups of sensitized guinea-pigs and the animals were exposed to the antigen aerosol after either 48 hr or one week of treatment. Control groups received corresponding treatment with arachis oil, or saline, and were exposed to the antigen aerosol after similar periods. Preconvulsion times were noted (Table 1).

TABLE 1. EFFECTS OF REPEATED DAILY ADMINISTRATION OF INHIBITORS OF ADRENOCORTICAL SECRETION ON ANAPHYLACTIC PRECONVULSION TIMES IN THE GUINEA-PIG. Preconvulsion times expressed as means for groups of five animals ± standard errors.

Treatment (daily intramuscular injections)	Preconvulsion times (sec) (means ± s.e.)	
	48 hr	1 week
DDD (50 mg/kg)	35·0 ± 2·0	17·8 ± 2·9
Controls	46·2 ± 2·2*	49·2 ± 2·5*
Amphenone-B (200 mg/kg)	35·5 ± 4·2	39·8 ± 3·8
Controls	53·6 ± 4·0*	47·4 ± 2·7
Metyrapone (200 mg/kg)	42·8 ± 3·5	39·0 ± 3·8
Controls	63·4 ± 5·4*	56·4 ± 2·9*

* Denotes significant difference $P < 0·05$.

The repeated administration of either DDD, amphenone-B, or metyrapone over 48 hr caused significant decreases in anaphylactic preconvulsion times compared with the control groups. Preconvulsion times were significantly decreased after treatment with DDD or metyrapone for one week, but not after amphenone-B.

Guinea-pigs undergoing these treatments for one week were weighed initially and then immediately before exposure to the antigen aerosol. After induction of anaphylactic reactions the animals were killed and the adrenal glands removed and weighed. The effects of the treatment on body weight and upon adrenal gland weights are in Table 2. Animals treated with DDD had decreased body weight and weight of adrenal glands. The mean weights of adrenal glands of animals receiving either

TABLE 2. EFFECTS OF REPEATED DAILY ADMINISTRATION OF ADRENOCORTICAL INHIBITORS ON TOTAL BODY WEIGHT AND ADRENAL GLAND WEIGHT IN THE GUINEA-PIG. Each value represents the mean weight from a group of five animals.

Treatment (daily intramuscular injections)	Mean total body weight (g)			Adrenal weight (g)	
	Initial	Final	% change	Mean	% of control
DDD (50 mg/kg)	432	421	−2·5	0·43	87
Controls	445	469	+5·4	0·49	
Amphenone-B (200 mg/kg) ..	391	398	+1·8	0·54	110
Controls	421	444	+5·7	0·49	
Metyrapone (200 mg/kg) ..	426	427	+0·2	0·65	118
Controls	420	444	+5·7	0·52	

amphenone-B or metyrapone were greater than those of the controls, but body weight increases were less. When examined before exposure to the antigen aerosol, the animals treated with adrenal inhibitors gave the subjective impression of being in poor physical condition, showing muscular weakness by apparent lack of limb and posture control, with dirtiness and lack of sheen of the coat. This evidence was taken as a general indication that effective adrenocortical inhibitor action had been produced.

TIME-COURSE OF THE EFFECT OF REPEATED ADMINISTRATION OF METYRAPONE

Metyrapone (200 mg/kg, s.c.) was administered at 12-hrly intervals to groups of sensitized guinea-pigs, and the animals were exposed to the antigen aerosol at 6, 12, 24, 48 or 96 hr after treatment began. Corresponding saline-treated control groups were exposed after similar times. Preconvulsion times are in Table 3. Repeated administration of metyrapone produced a gradual decrease in anaphylactic preconvulsion times, significant differences being apparent after 48 and 96 hr.

TABLE 3. THE EFFECTS OF REPEATED ADMINISTRATION OF METYRAPONE (200 MG/KG, S.C. AT 12 HR INTERVALS) ON ANAPHYLACTIC PRECONVULSION TIMES OF SENSITIZED GUINEA-PIGS. Mean results from groups of five animals ± standard errors.

Duration of treatment (hr)	Preconvulsion times (sec) (mean ± s.e.)		% decrease
	Control	Test	
6	57·6 ± 2·8	56·8 ± 2·2	1·4
12	45·8 ± 1·4	40·8 ± 2·7	10·9
24	67·6 ± 4·3	61·2 ± 2·9	9·4
48	66·1 ± 4·7	52·8 ± 3·0	20·0*
96	48·5 ± 2·3	33·0 ± 4·0	32·0*

* Denotes significant differences $P < 0{\cdot}05$

EFFECT OF METYRAPONE IN COMBINATION WITH CORTICOTROPHIN

With an interval of 12 hr, two subcutaneous doses of either metyrapone (200 mg/kg) alone, corticotrophin (10 u/kg) alone, or both simultaneously, were administered to groups of sensitized guinea-pigs. Twelve hr after the last injection, the animals were exposed to the antigen aerosol, and preconvulsion times were measured. Saline-treated control groups were similarly exposed. Results are in Table 4.

TABLE 4. THE EFFECTS OF METYRAPONE (200 MG/KG, 2 × 12 HRLY, S.C.) AND CORTICOTROPHIN (10 U/KG, 2 × 12 HRLY S.C.) ON ANAPHYLACTIC PRECONVULSION TIMES IN THE GUINEA-PIG. Preconvulsion times expressed as mean ± standard error.

Treatment	Number of animals	Preconvulsion times (sec) (mean ± s.e.)	
		Control	Test
Metyrapone	5	53·6 ± 3·8	47·0 ± 1·2
ACTH	6	46·1 ± 5·5	48·6 ± 5·1
Metyrapone + ACTH	5	53·6 ± 3·8	37·8 ± 4·9*

* Denotes significant difference P < 0·05.

No significant difference from the controls was observed in animals treated with metyrapone or with corticotrophin alone. However, animals treated with metyrapone and corticotrophin showed a further and significant decrease in preconvulsion times, thus indicating a potentiating effect of the combination.

EFFECTS OF MINERALOCORTICOID DRUGS

Groups of sensitized guinea-pigs treated with single doses of either deoxycortone (10 mg/kg, i.m.), cortexolone (5 mg/kg, i.m.) or aldosterone (1·0 mg/kg, i.m.), were exposed to the antigen aerosol after 4, 8, 12 or 24 hr. Preconvulsion times for these and for corresponding saline or arachis oil treated control groups are in Table 5.

TABLE 5. ANAPHYLACTIC PRECONVULSION TIMES OF SENSITIZED GUINEA-PIGS AT VARIOUS TIMES AFTER ADMINISTRATION OF SINGLE DOSES OF DEOXYCORTONE (10·0 MG/KG, I.M.), CORTEXOLONE (5·0 MG/KG, I.M.) ALDOSTERONE (1·0 MG/KG, I.M.) OR FLUDROCORTISONE (4·0 MG/KG, I.M.). Results are expressed as mean values for groups of five animals ± standard errors.

Treatment	Interval (hr)	Preconvulsion times (sec) (mean ± s.e.)	
		Control	Test
Deoxycortone	4	50·4 ± 2·4	36·0 ± 2·6*
	8	52·6 ± 3·8	40·1 ± 1·2*
	12	46·5 ± 7·8	44·1 ± 5·6
	24	49·2 ± 8·1	47·0 ± 9·8
Cortexolone	4	41·1 ± 3·4	26·5 ± 3·3*
	8	47·1 ± 3·2	38·2 ± 5·0
	12	50·5 ± 8·3	54·2 ± 8·3
Aldosterone	4	54·2 ± 4·1	35·8 ± 3·4*
	8	45·6 ± 1·8	44·7 ± 2·1
	12	37·0 ± 6·7	33·0 ± 1·8
	24	42·5 ± 3·2	43·8 ± 2·7
Fludrocortisone	4	32·2 ± 1·9	32·5 ± 3·1
	18	37·2 ± 3·1	58·2 ± 5·6*

* Denotes significant difference P < 0·05.

In animals exposed to the antigen 4 hr after these mineralocorticoid drugs, preconvulsion times were significantly lower. Such a decrease was also observed after 8 hr in deoxycortone-treated animals. No significant differences were observed after longer times.

Single intramuscular injections of fludrocortisone (4mg/kg) were administered to sensitized guinea-pigs and anaphylactic reactions were

induced 4 or 18 hr later. Preconvulsion times (Table 5) were compared with those from sensitized animals treated with the injection vehicle.

No significant difference in preconvulsion times resulted from fludrocortisone treatment 4 hr before the reaction. In the guinea-pigs exposed 18 hr after fludrocortisone there was a significant increase in preconvulsion times.

EFFECTS OF MEPYRAMINE ON ANIMALS TREATED WITH METYRAPONE OR CORTICOSTEROIDS

Six sensitized guinea-pigs were treated with 4 subcutaneous doses of metyrapone (200 mg/kg) at 12 hrly intervals, and each animal was exposed to the antigen aerosol 12 hr after the last dose. Other groups of six animals were treated with single doses of aldosterone (1·0 mg/kg, i.m.), or cortexolone (5·0 mg/kg, i.m.), and exposed to the antigen aerosol 4 hr after treatment. One group of animals was treated with fludrocortisone (4 mg/kg, i.m.), and anaphylactic reactions were induced 18 hr later. In all of these animals a single subcutaneous dose of mepyramine (1·0 mg/kg) was administered 1 hr before exposure to the aerosol. Preconvulsion times were recorded, and compared with those from corresponding mepyramine-treated control animals (Table 6).

TABLE 6. THE EFFECTS OF METYRAPONE AND CORTICOSTEROIDS ON ANAPHYLACTIC PRECONVULSION IN MEPYRAMINE-TREATED GUINEA-PIGS (1·0 MG/KG, S.C., 1 HR). Six animals per group.

Treatment	Preconvulsion times (means ± s.e.) (sec)		% change
	Control mepyramine alone	Test	
Metyrapone (4 × 12 hrly) 200 mg/kg, s.c. ..	189 ± 17	125 ± 15	33·9 decrease*
Aldosterone (4 hr) 1·0 mg/kg	151 ± 23	133 ± 15	12·0
Deoxycortone (4 hr) 10·0 mg/kg	151 ± 23	118 ± 13	21·8
Cortexolone (4 hr) 5·0 mg/kg	151 ± 23	168 ± 18	11·2
Dexamethasone (18 hr) 4·0 mg/kg	177 ± 14	409 ± 49	131·8* prolongation
Fludrocortisone (18 hr) 4·0 mg/kg	177 ± 14	340 ± 34	92·2* prolongation

* Denotes significant difference P < 0·05.

Preconvulsion times of animals treated with aldosterone, cortexolone or deoxycortone, followed by mepyramine, were not significantly different from animals receiving mepyramine alone. In metyrapone-treated guinea-pigs given mepyramine, preconvulsion times were significantly decreased. On the other hand, the mean preconvulsion time of the groups treated with mepyramine and fludrocortisone was significantly longer than that of the control group treated with mepyramine alone. In this case the prolongation of preconvulsion time by fludrocortisone previously administered alone, was much enhanced by mepyramine.

Thus mepyramine prevented the decreases in preconvulsion time caused by aldosterone, cortexolone and deoxycortone, but did not modify the effect of metyrapone. It potentiated the prolongation of preconvulsion times by fludrocortisone.

Discussion

A survey of the literature indicated that a protective effect of corticosteroid drugs may be demonstrated using preconvulsion-time methods for evaluation of aerosol-induced anaphylaxis (Herxheimer & Rosa, 1952; Mendes, 1957; Goadby & Smith, 1964; Gorog & Szporny, 1965).

Adrenalectomy results in increased severity of anaphylactic reactions in many species, including the rabbit, dog, rat and mouse (Rose, 1959). The lack of clear-cut evidence that adrenalectomy has a similar result in the guinea-pig is therefore anomalous. It would be expected that a lack of adrenocortical secretion, a restraining influence on anaphylaxis in the normal animal, should similarly potentiate anaphylactic severity.

The experiments have shown that the repeated daily administration of either DDD or metyrapone for 48 hr or one week increased the severity of anaphylaxis, as indicated by significantly shortened preconvulsion times. The degree of adrenocortical deficiency was not evaluated but during these treatments signs consistent with such deficiency were observed: poor condition, possible muscular weakness, and particularly the failure to gain body weight. Furthermore, the mean weights of adrenal glands in DDD-treated animals were less than, whereas those from metyrapone-treated animals were more than, the corresponding controls. Inhibition of corticosteroid secretion by DDD treatment is associated with adrenal cortical atrophy (Nelson & Woodward, 1947; Nichols & Sheehan, 1952), whereas inhibition of corticosteroid secretion by metyrapone treatment is associated with adrenal hypertrophy (Chart & Sheppard, 1959).

A decrease in anaphylactic preconvulsion times was also observed after treatment with amphenone-B for two days, but was less marked after its administration for one week. This compound is a potent inhibitor of adrenocortical secretion in most species, but its effects are less potent in the guinea-pig (Chart & Sheppard, 1959) and it has a wide spectrum of biological activity. It is possible, therefore, that its effect on adrenocortical secretion is masked for instance by its potent antithyroid properties, which may be expected to decrease anaphylactic severity.

The one common property of DDD, metyrapone and amphenone-B is the inhibition of corticosteroid secretion, and it is therefore probable that this is the cause of the decreased anaphylactic preconvulsion times.

The present findings have shown that corticotrophin potentiated the effects of metyrapone, whereas corticotrophin administered alone had no such effect. Chart & Sheppard (1959) have shown that the inhibition of secretion of all 17-hydroxycorticoids by metyrapone, and the consequent increase in adenohypophysial corticotrophin secretion, results in adrenal hypertrophy. The increased corticotrophin levels also further stimulate corticosteroid biosynthesis. Metyrapone basically inhibits 11β-hydroxylation of corticosteroids in the final stages of biosynthesis, so that under the influence of corticotrophin an accumulation and secretion of steroid precursors, the 11-desoxysteroids cortexolone and deoxycortone, occurs. The largely glucocorticoid nature of the normal adrenocortical secretion is thus replaced by steroids with a predominantly mineralocorticoid nature.

The effects of administration of mineralocorticoid substances on anaphylaxis were therefore investigated, because an increase in this type of activity could contribute to the potentiation of the effects of metyrapone by corticotrophin. Mineralocorticoids were found to potentiate anaphylaxis.

Investigations of the normal secretion from the guinea-pig adrenal cortex indicate that the predominant secretion is hydrocortisone (Bush, 1962) and resting blood concentrations of corticosteroids in this species are higher than most other mammals (Done, Ely, & others, 1952). Thus the decreased anaphylactic preconvulsion times caused by metyrapone in the present work could be explained by a deficiency of glucocorticoid secretion.

Under the influence of metyrapone the anaphylaxis-potentiating effect of the mineralocorticoids which replace the predominantly glucocorticoid hydrocortisone secretion, may be expected to contribute further to the effect on anaphylaxis. It is of considerable interest that a large dose of the potent mineralocorticoid fludrocortisone failed to decrease anaphylactic preconvulsion times after 4 hr. But this compound, which also possessed marked glucocorticoid and anti-inflammatory properties, significantly prolonged preconvulsion times of reactions induced 18 hr after its administration. Significant anti-anaphylactic effects of potent anti-inflammatory steroids are observed when these are administered an optimal 18 hr before anaphylaxis (Goadby & Smith, 1964; Feinburg & Malkiel, 1952).

The combined effects of fludrocortisone and mepyramine much prolonged anaphylactic preconvulsion times, and the result was greater than that of a summation of their individual effects. This is comparable with the findings of Winter & Flataker (1955), and Goadby & Smith (1964), who showed that cortisone and related compounds had only small protective effects against anaphylaxis in the guinea-pig, but markedly potentiated mepyramine. This is further evidence that the observed effects of fludrocortisone are related to its glucocorticoid-anti-inflammatory activity rather than its mineralocorticoid properties. In contrast mepyramine pretreatment prevented the decrease in preconvulsion times caused by the mineralocorticoids.

In the mepyramine-treated animals it may be assumed that the contribution of histamine to the total anaphylactic response is so reduced that the observed reaction is mediated largely by the non-histamine component (Goadby & Smith, 1964). Thus any effects of corticosteroid treatment in such animals may be considered to arise from an influence on the residual response induced by SRS-A and probably other active materials. As pretreatment with mepyramine prevented the decrease in preconvulsion times resulting from mineralocorticoid administration, it is suggested that these steroids influenced mainly the histaminic component of the anaphylactic reaction. Such effects may be related to the increase in tissue histamine levels accompanying the administration of mineralocorticoids in the guinea-pig (Hicks, 1965).

Treatment of the guinea-pigs with repeated injections of metyrapone, followed by a single dose of mepyramine, resulted in a significant decrease

in anaphylactic preconvulsion times, and it would thus appear that at least part of the effect of metyrapone is exerted on the residual non-histaminic component. This evidence is consistent with a suggestion that the main effect of metyrapone results from deficiency of the normal predominantly glucocorticoid secretion. The relative dimensions of any contribution due to the replacement of this secretion by mineralocorticoids is difficult to assess, without a more precise knowledge of actual levels of secretion. The potentiation of the metyrapone action by corticotrophin suggests that the mineralocorticoid contribution could be an important component of the overall effect. It is of interest to note that administration of metyrapone provoked an increase in tissue histamine levels in the guinea-pig (Kovacs, 1965; Hicks, 1965), and that this effect is potentiated by simultaneous administration of corticotrophin.

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The colloidal properties of chlorhexidine and its interaction with some macromolecules

D. D. HEARD AND (IN PART) R. W. ASHWORTH

Surface tension, conductivity and dye solubilization experiments show that, in aqueous solution, chlorhexidine diacetate forms micelles and has a molar critical micellar concentration (CMC) of 0·010–0·011 at 25°. Similarly, the digluconate salt has a molar CMC of 0·0066. Above its CMC, the freely soluble digluconate salt solubilizes the less soluble diacetate salt. The formation of micelles does not affect the rate of hydrolysis of chlorhexidine to *p*-chloroaniline. It is suggested that the micelles formed by chlorhexidine resemble those formed by many dyes rather than those of colloidal surfactants. Equilibrium dialysis experiments show that 1·0 and 3·0% polysorbate 80 inactivates 37·5 and 70·0% respectively of the chlorhexidine in a 0·10% solution of the diacetate salt.

A SIMILARITY in the mode of action between the potent antibacterial compound chlorhexidine (Hibitane) and the quaternary ammonium germicides has been pointed out by Hugo & Longworth (1964) and Rye & Wiseman (1964). Also it is well known that aqueous solutions of chlorhexidine froth markedly on gentle agitation. These two facts have prompted an investigation to see if chlorhexidine behaves as a typical surface-active agent, and, in particular, if it forms micelles and shows a critical micelle concentration (CMC). In addition, chlorhexidine, like other antibacterial compounds, is known to be less active in the presence of non-ionic surfactants. As no quantitative data were available, physico-chemical studies of the degree of inactivation have been made.

SURFACE AND COLLOID PROPERTIES

Determinations of CMC were made using a commercial sample of the diacetate salt, but the digluconate solutions were prepared from recrystallized base and 1,5-gluconolactone solutions. Deionized water was used throughout.

Surface tension measurements were made using a Du Nouy tensiometer at room temperature in the usual way, except that surface-ageing effects made it necessary to form the surface 24 hr before measurement. The corrections of Harkins & Jordan (1930) were applied. The results are shown in Fig. 1. A Mullard conductivity bridge and a dipping electrode were used at 25° (±0·01°) for the conductivity measurements. These results are shown in Fig. 2. The standard procedure was followed for determining the CMC by dye solubilization (Rigg & Liu, 1953). Agitation was for 7 days at 25° (±0·1°). Satisfactory results could not be obtained for the digluconate salt but those for the diacetate are shown in Fig. 3. The CMC values obtained by the above methods are listed in Table 1. The presence of large aggregates in chlorhexidine diacetate solutions above the CMC and their absence below was demonstrated by the analytical ultracentrifuge.

The solubilization of chlorhexidine diacetate by the very soluble digluconate was determined by shaking excess diacetate salt with solutions

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of the digluconate of known concentration at 25° ($\pm$0·1°). Aliquots were filtered after equilibration (48 hr) and the total chlorhexidine content of the filtrate determined colorimetrically (Holbrook, 1958). The increase in chlorhexidine content must be due to the diacetate salt and Fig. 4 shows diacetate solubility at various concentrations of digluconate.

Experiments to determine the effect of chlorhexidine concentration on the rate of *p*-chloroaniline formation were also made. The *p*-chloroaniline contents of various concentrations of chlorhexidine diacetate, chosen to

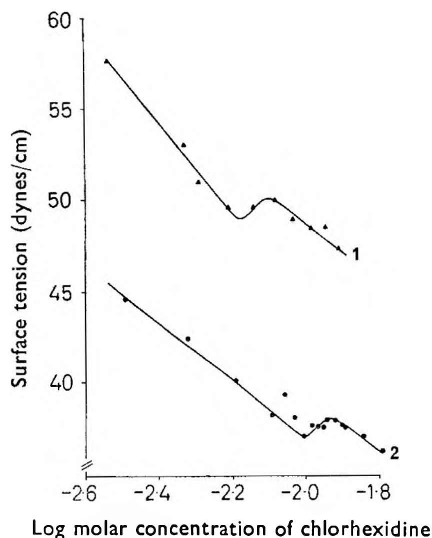

FIG. 1. Determination of CMC values of (1) chlorhexidine digluconate, (2) chlorhexidine diacetate, by surface tension measurements at room temperature

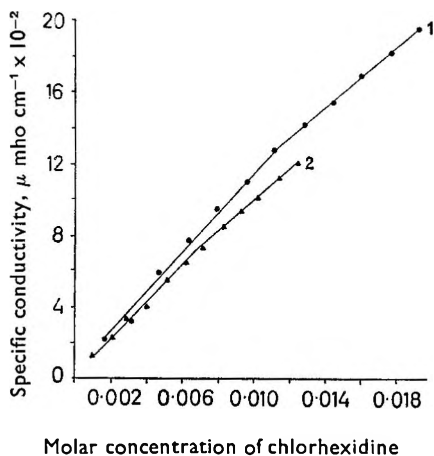

FIG. 2. Determination of CMC values of (1) chlorhexidine diacetate and (2) chlorhexidine digluconate by conductivity measurements at 25°.

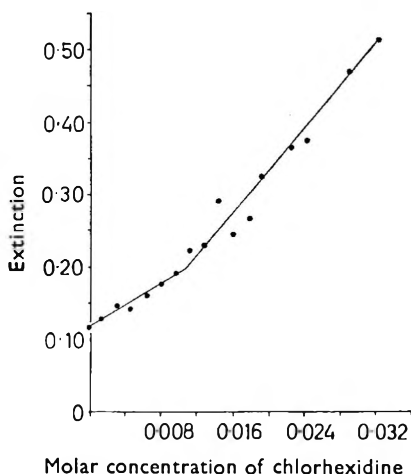

FIG. 3. Determination of CMC of chlorhexidine diacetate by dye solubilization at 25°.

be above and below the CMC (0·77%) at the temperature of the experiment (80°), were determined by a method based on that in the B.P. 1963. Two series of ampoules were filled with the solutions and stored at 80° for 64 and 136 hr respectively. The *p*-chloroaniline concentration was redetermined.

TABLE 1. THE CMC OF CHLORHEXIDINE DIGLUCONATE AND DIACETATE AS DETERMINED BY VARIOUS METHODS

Chlorhexidine salt	Method	CMC Molar	CMC % w/v
Diacetate	Surface tension	0·010	0·63
Diacetate	Conductivity	0·011	0·69
Diacetate	Solubilization	0·0105–0·011	0·66–0·69
Digluconate ..	Surface tension	0·0066	0·59
Digluconate ..	Conductivity	0·0066	0·59

INTERACTION OF CHLORHEXIDINE DIACETATE WITH MACROMOLECULES

To investigate the interaction between chlorhexidine diacetate and polysorbate (Tween) 80 (Honeywill-Atlas), Visking dialysis tubing (Scientific Instrument Centre) was suitable provided that the dialysis was not allowed to continue beyond the time necessary for equilibration of the chlorhexidine diacetate, thus keeping negligible the error caused by dialysis of the surfactant. Dialysis bags, just large enough to hold 20 ml of surfactant solution, were prepared and immersed in 20 ml of chlorhexidine diacetate solution contained in glass-stoppered jars. The jars were agitated at 25° for 5 hr for solutions containing up to 0·2% chlorhexidine diacetate and for 18 hr for stronger solutions. At equilibrium the chlorhexidine content on both sides of the membrane was determined either by measuring the extinction at 254 mμ after suitable dilution or colorimetrically (Holbrook, 1958). When the former method was used, correction for absorbance by the surfactant was necessary. The

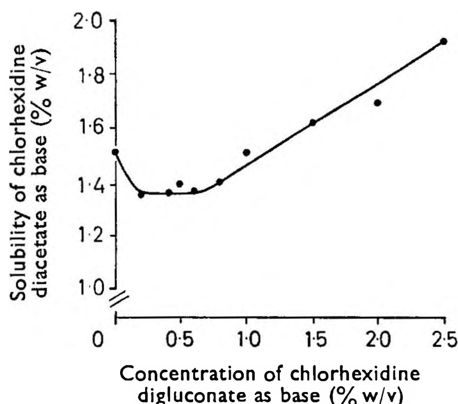

FIG. 4. Effect of chlorhexidine digluconate concentration on the solubility of chlorhexidine diacetate at 25°.

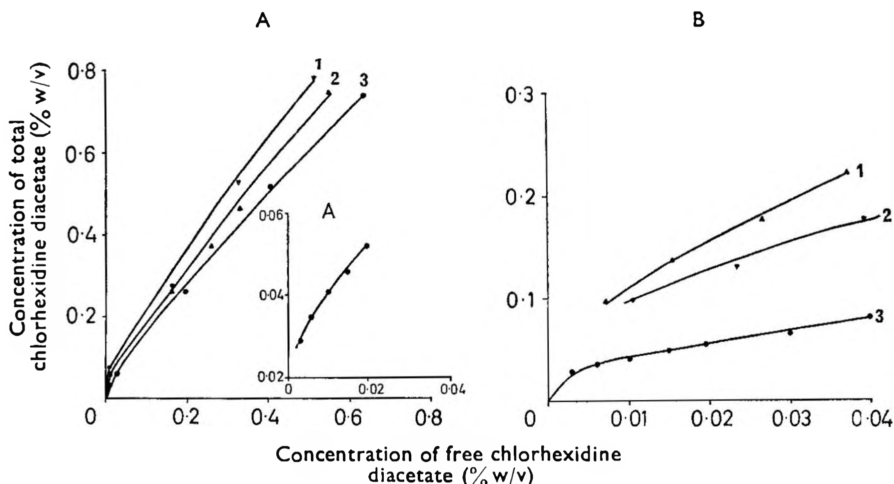

FIG. 5A. A plot showing the interaction of chlorhexidine diacetate with (1) 3%, (2) 2%, (3) 1% polysorbate 80 at 25°. A is a plot of the interaction of 1% polysorbate 80 at low chlorhexidine diacetate concentrations.

B. A plot showing the effect of various concentrations of sodium acetate on the interaction of chlorhexidine diacetate with polysorbate 80 at 25° and pH 5·8. (1) 0·25 M buffer, (2) 0·1 M buffer, (3) buffer absent.

results for various polysorbate 80 concentrations are in Fig. 5A which shows the amount of free chlorhexidine diacetate at various total concentrations. The dialysis experiments were repeated in the presence of 0·1 and 0·25M sodium acetate buffers respectively at pH 5·8 (this is the pH of unbuffered chlorhexidine diacetate and polysorbate 80 mixtures). The results are in Fig. 5B.

Because ethanol has a dis-aggregating effect on non-ionic surfactant micelles (Becher, 1965) the dialysis experiments were again repeated in the presence of various concentrations of ethanol. The solubility of

chlorhexidine diacetate in ethanol–water mixtures and the effect of ethanol concentration on the CMC of polysorbate 80, determined by the method of Becher (1962), are shown in Table 2.

TABLE 2. THE EFFECT OF VARIOUS CONCENTRATIONS OF AQUEOUS ETHANOL ON THE SOLUBILITY OF CHLORHEXIDINE DIACETATE AND THE CMC OF POLYSORBATE 80

Concentration of ethanol % v/v	Solubility of chlorhexidine diacetate at 25°	CMC polysorbate 80 at 20°
0	1·89% w/v	0·0078% w/v
10	2·40	0·0105
20	3·65	0·0145
30	—	0·0263
35	—	0·053
40	—	No micelles formed
50	14·40	—
60	18·40	—

Using the same dialysis technique, the interaction of chlorhexidine diacetate with both methylcellulose and polyvinylpyrrolidone was determined. At equilibrium, the concentrations of chlorhexidine on both sides of the membrane were essentially the same.

Discussion

SURFACE AND COLLOID PROPERTIES

Surface tension, conductivity and dye solubilization experiments all show that at a certain concentration, aqueous solutions of chlorhexidine exhibit a sharp change in the relevant physico-chemical property. The concentration at which this occurs depends on the counter-ion. This is strong evidence that aggregation of either single molecules or, possibly, small aggregations of molecules are starting to form micelles at this concentration. Confirmation of this comes from the analytical ultracentrifuge which demonstrated that only above these concentrations were large aggregates present. Chlorhexidine consists of a series of alternate hydrophilic and hydrophobic groups. In no way can it be considered to have an amphipathic character in the sense of having a polar head and a non-polar chain. It is suggested, therefore, that like polyvinyl alcohol, chlorhexidine should be considered as a specific surface-active agent (Moilliet, Collie & Black, 1961a). Thus the reduction in surface tension is caused by some specific group being attracted to the air-water interface.

Similarly, it is difficult to visualize how chlorhexidine could form a micelle of the type formed by colloidal surfactants. It has been known for many years that water-soluble dyestuffs often exist in solution in an aggregated form (Vickerstaff, 1954). Aggregation of these dyes is not necessarily associated with adsorption at the air–water interface (Alexander & Stacey, 1952) and it has been suggested that aggregation is primarily due to some specific forces, in particular those due to hydrogen bonding, rather than amphipathy in the dyestuff molecule (Moilliet, Collie & Black, 1961b). We hypothesize that the aggregation of chlorhexidine to form micelles is more akin to the aggregates formed by dyestuffs

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than by colloidal surfactants and that the force responsible may be hydrogen-bonding associated with the diguanido-groups. Figs 1 and 2 both show the effect of the counter-ion on the CMC of chlorhexidine. It is well known that the counter-ion can alter the CMC and this is usually associated with a change in micellar size.

Klevens (1950) gives several examples where the micelles of a more soluble salt have solubilized a less soluble salt. This is clearly shown by chlorhexidine digluconate and diacetate in Fig. 4. Two points emerge from these results. Firstly, the concentration at which solubilization commences (0·60–0·80% of chlorhexidine base) is slightly higher than the CMC of chlorhexidine digluconate as determined by surface tension and conductivity measurements (0·59%) and, secondly, below the CMC there is a slight, but definite, decrease of solubility of chlorhexidine diacetate compared with its solubility in water. This might be expected from solubility product considerations.

Chlorhexidine slowly hydrolyses in aqueous solution to give, among other products, *p*-chloroaniline (Goldman, J. & Goodall, R. R., unpublished observation). The formation of micelles might lead to some protection of the hydrolysable group and thus reduce the rate of hydrolysis, but for the conditions and concentrations studied, no change in hydrolysis rate above and below the CMC could be detected [cf. sodium lauryl sulphate (Nogami, Awazu & Kanakubo, 1963)].

Hugo & Longworth (1964) and Rye & Wiseman (1964) have pointed out similarities between the mode of action of chlorhexidine and the quaternary ammonium antibacterial compounds. The fact that these latter compounds and chlorhexidine lower the surface tension of water and form micelles is a further similarity. However, it must be remembered that although at the CMC the surface tension of cetrimide solutions is similar to that of chlorhexidine diacetate solutions, the concentration of the former is lower, on a molar basis, by about one order of magnitude.

INTERACTION WITH MACROMOLECULES

Fig. 5A shows that like many other antibacterial compounds, chlorhexidine interacts with polysorbate 80. The higher the concentration of the polysorbate 80, the greater is the interaction. Also, the ratio of free to total chlorhexidine is dependent at low chlorhexidine concentrations on the actual concentration of chlorhexidine present. This is shown more clearly in insert A, Fig. 5A. Table 3 compares the inactivation of

TABLE 3. COMPARISON OF THE INACTIVATION OF CHLORHEXIDINE DIACETATE BY POLYSORBATE 80 AS DETERMINED BY A DIALYSIS METHOD AND A BACTERICIDAL TECHNIQUE USING *Staph. aureus*

Concentration polysorbate 80	Method	% inactivation of 0·1% chlorhexidine diacetate
1·0	Dialysis at 25°	37·5
1·0	Bactericidal at 30°	61·0
3·0	Dialysis at 25°	70·0
3·3	Bactericidal at 30°	86·0

chlorhexidine diacetate by polysorbate 80 as determined in this present work with the inactivation as determined by an *in vitro* bactericidal technique using *Staphylococcus aureus* (Mr. R. Hall, unpublished observations).

There are a number of possible reasons for the agreement between the two methods not being closer. The two series of experiments were made at different temperatures, concentrations of polysorbate 80 were slightly different and replaced thermodynamic activities, and no allowance was made for possible interference from the Donnan effect. Closer examination shows that the effects of all these factors are likely to be small. A more likely reason for the difference is that whilst the physico-chemical technique only measures the interaction of the surfactant with chlorhexidine, the bactericidal test in addition measures any interference by the surfactant on the uptake of chlorhexidine by the bacteria (Wedderburn, 1964).

Fig. 5B shows the effect of various concentrations of acetate ion on the interaction between chlorhexidine and polysorbate 80. Acetate ion might be expected to decrease the solubility of chlorhexidine diacetate leading to increased interaction with the polysorbate 80. Acetate ion would also be expected to affect the polysorbate 80. Thus it might change the micellar size and lower the CMC. It is not possible to predict the magnitude of these changes (Elworthy & Macfarlane, 1965). However, the CMC of polysorbate 80 is so low (0·078% w/v) that reduction of this value could not increase the amount of micellar material sufficiently to account for the increase in interaction observed. The major effect leading to increased interaction between chlorhexidine diacetate and polysorbate 80 in the presence of excess acetate ion is, therefore, mainly due to the "salting-out" of the chlorhexidine.

The effects of ethanol on the aqueous solubility of chlorhexidine diacetate and the CMC of polysorbate 80 are shown in Table 2. There is a fall in interaction between chlorhexidine diacetate and polysorbate 80 with increasing concentrations of ethanol. The fall in interaction in the presence of 10 and 20% of ethanol is probably more a result of the increased solubility of chlorhexidine diacetate in the aqueous phase than of the slight reduction in micellar surfactant. That interaction still occurs between chlorhexidine diacetate and polysorbate 80 in the presence of 50 and 60% of ethanol when no micelles of surfactant are present must mean that chlorhexidine diacetate can interact with monomeric polysorbate 80 in the presence of aqueous ethanol. As with phenols (Mulley & Metcalf, 1956), this interaction may well be hydrogen-bonding between the diguanido-groups and the ether oxygens of the polyoxyethylene chains. Because the polysorbate 80 is in a non-micellized form in the presence of the two higher concentrations of ethanol, it will diffuse more rapidly through the dialysis membrane. This will result in some error associated with the interaction values at these concentrations.

There are essentially three areas in a micelle where a compound can be solubilized (Riegelman, Allawala & others, 1958). These are the central portion, the surface of the micelle and the intermediate "palisade" layer. Chlorhexidine is essentially insoluble in hydrocarbon solvents and we consider that, like dimethyl phthalate, it is probably adsorbed on the

surface of the micelle. A suggestion for the shape of the interaction curves in Fig. 5A can now be made. Fig. 5A shows relatively much greater interaction at low chlorhexidine concentrations, followed by a roughly linear relation between total and free chlorhexidine. This could be caused by the two different types of interaction, namely adsorption on the micellar surface and hydrogen-bonding with the polyoxyethylene chain, which it is postulated chlorhexidine diacetate and polysorbate 80 can undergo.

Both methylcellulose and polyvinylpyrrolidone with chlorhexidine diacetate gave equilibrium concentrations which were essentially equal on both sides of the membrane. From this it follows that chlorhexidine diacetate interacts with neither. Miyawaki, Patel & Kostenbauder (1959) have previously shown that methyl- and propyl-*p*-hydroxybenzoates interact with both methylcellulose and polyvinylpyrrolidone. However, neither of the quaternary ammonium compounds tested by Deluca & Kostenbauder (1960) interacted with polyvinylpyrrolidone although one, cetylpyridinium chloride, did interact with methylcellulose.

The experimental work reported here shows that in common with other antibacterial agents, chlorhexidine shows some inactivation by polysorbate 80. Although the amount of inactivation is not so large as with certain other antibacterials, nevertheless, for optimum activity the concentration of non-ionic surfactant should be kept as low as practicable.

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The effect of the concentration of the water-soluble component on the rheology of some oil-in-water emulsions containing long-chain alcohols

F. A. J. TALMAN, P. J. DAVIES and (MISS) E. M. ROWAN

The effect of the concentration of two non-ionic water-soluble components—Sorbester Q12 (polyoxyethylene sorbitan monolaurate) and cetomacrogol 1000—and two ionic water-soluble components—cetrimide and sodium lauryl sulphate—on the rheology of some emulsions has been examined. Emulsions containing oleyl and lauryl alcohol exhibited an increase in consistency with increasing surfactant content. Maximum values for static yield value and apparent viscosity were found to occur with cetostearyl alcohol as the oil-soluble component. The values of the rheological parameters for products containing ionic surfactants tended to be higher than for non-ionics, but molal plots (and preliminary studies with Texofor N4, Texofor FX170 and Solumin FX170SD) suggested that this was an effect due to molecular weight rather than charge.

IT has previously been postulated by Talman, Davies & Rowan (1967) that the flow behaviour of a series of oil-in-water emulsions was related to the "intrinsic" strength of a gel formed in the continuous phase by the interaction of oil- and water-soluble components. The present paper is concerned with the effect of water-soluble component concentration on a similar series of emulsions.

Experimental

The general formula used for the preparation of the emulsions was: liquid paraffin, 50·0, oil-soluble component, 0·25 to 10, water-soluble component, 0·125 to 5·0, distilled water to 100·0 g.

The materials and methods given below are additional to those previously described (Talman & others, 1967).

Materials. An ethoxylated alkyl cresol (Texofor FX170), the sodium salt of the corresponding sulphated material (Solumin FX170SD) and an ethoxylated aliphatic alcohol (Texofor N4) [from Glovers (Chemicals) Ltd., Leeds] were used without further purification.

Preparation of gels. The technique adopted was similar to that employed for the emulsions. Since the latter had a phase weight ratio of about 0·5, the concentration of water-soluble component used to prepare a gel was twice that of the corresponding emulsion. All gels contained 10% w/w of the stated alcohol.

Rheological examination. Boylan (1966) has examined a number of pharmaceutical products using a Ferranti-Shirley viscometer and has pointed out some of the difficulties inherent in meaningful interpretation of the rheograms. The spur or abrupt change of slope which was characteristic of the rheograms for which we have reported a static yield value, was replaced in some instances by a more gradual change of slope. For comparative purposes, in the latter case we took the mean of the shear stress range over which the gradual change occurred and quote this as the mean static yield value in Tables 1, 3 and 5.

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Results and discussion

With emulsions containing oleyl alcohol, an increase in the concentration of the water-soluble component generally resulted in the appearance of pseudoplasticity and a rise in apparent viscosity. The latter was more marked in those emulsions containing the higher concentrations of the alcohol in combination with ionic surfactants (Table 1). Oleyl alcohol dispersed in solutions of water-soluble components to give thin milky fluids rather than gels, an exception being 5·0% w/w cetrimide solution. In general, the onset of pseudoplasticity and rise in apparent viscosity cannot therefore be attributed to even weak gel formation. The slight increase in viscosity of the external phase (1 to 3cP for 0·5 to 5·0% w/w cetomacrogol 1000), accentuated by thin film effects in the interglobular spaces and electroviscous phenomena probably account for the observed changes (Sherman, 1964). We found that at constant amounts of water-soluble component the alterations in consistency with increasing oleyl alcohol content were largely due to phase weight ratio effects. Where a gel was formed, as with 5·0% w/w cetrimide, the emulsions containing 8 and 10% w/w oil-soluble component exhibited a mean static yield value.

TABLE 1. EFFECT OF WATER-SOLUBLE COMPONENT CONCENTRATION ON APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF EMULSIONS CONTAINING OLEYL ALCOHOL

Water-soluble component		Oleyl alcohol concentration (% w/w)											
		1		2		4		6		8		10	
	% w/w	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV
Cetomacrogol 1000	0·5	11	0	16	0	18	0	21	0	25	0	27	0
	1·0	16	0	18	0	21	0	25	0	32	0	34	0
	2·0	18	0	20	0	24	0	30	0	37	0	47	*
	5·0	33	0	37	*	44	*	53	*	64	*	76	*
Sorbester Q12	0·5	10	0	11	0	13	0	15	0	18	0	22	0
	1·0	16	0	15	0	17	0	23	0	25	0	33	*
	2·0	17	0	18	0	20	0	27	0	32	*	39	*
	5·0	24	0	26	0	32	0	36	*	45	*	57	*
Cetrimide	0·5	13	0	11	0	13	0	14	0	20	0	21	0
	1·0	9	0	10	0	14	0	19	0	14	0	33	*
	2·0	9	0	9	0	17	0	29	*	39	*	52	*
	5·0	17	0	19	0	82	*	139	*	180	†1382	228	†1809
Sodium lauryl sulphate	0·5	15	0	11	0	15	0	19	0	23	0	30	0
	1·0	11	0	13	0	17	0	26	0	36	*	46	*
	2·0	6	0	9	0	19	0	32	*	63	*	63	*
	5·0	10	0	8	0	31	*	38	*	74	*	122	*

* Pseudoplastic.
† Mean static yield value.

The data for emulsions containing oleyl and lauryl alcohols, together with cetomacrogol 1000 or Sorbester Q12 as the water-soluble component, were remarkably similar. In contradistinction to oleyl alcohol, lauryl alcohol formed weak gels with both of the foregoing materials and might be expected to yield emulsions of slightly higher consistency than was actually observed. In separate experiments lauryl alcohol dissolved in liquid paraffin was carefully placed on top of solutions containing each of the water-soluble components. On standing at room temperature

there was no apparent change in those systems containing non-ionic surfactants whereas an amorphous precipitate formed in the aqueous phases containing sodium lauryl sulphate and cetrimide. It is significant that where there was no visible evidence of migration of alcohol from the oil to aqueous phase the emulsions were unexpectedly thin and did not have a static yield value. As may be seen from Table 2, nearly all emulsions containing an ionic material exhibited non-Newtonian flow characteristics; some having a static yield value. Larger amounts of both alcohol and ionic surfactant gave smooth products but the rheograms became so irregular that they could not be interpreted. Such behaviour was also apparent in gels prepared with cetrimide. We tentatively ascribe this irregularity to a large elastic component as has been noted by Pilpel (1966) for related systems.

TABLE 2. EFFECT OF WATER-SOLUBLE COMPONENT CONCENTRATION ON APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF EMULSIONS CONTAINING LAURYL ALCOHOL

Water-soluble component		Lauryl alcohol concentration (% w/w)											
		1		2		4		6		8		10	
	% w/w	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV
Cetomacrogol 1000.. ..	0·5	15	0	15	0	16	0	18	0	21	0	28	0
	1·0	16	0	17	0	20	0	24	0	29	0	34	*
	2·0	18	0	20	0	24	0	30	0	36	*	45	*
	5·0	28	0	22	0	46	*	55	*	65	*	83	*
Sorbester Q12	0·5	15	0	15	0	19	0	20	0	23	0	25	0
	1·0	13	0	15	0	17	0	22	0	25	0	31	*
	2·0	16	0	17	0	20	0	27	0	30	*	37	*
	5·0	25	0	27	0	32	0	39	*	43	*	59	*
Cetrimide ..	0·5	39	151	64	352	58	477	74	503	79	691	94	754
	1·0	36	*	88	452	127	302	202	979	249	1432	286	1709
	2·0	23	*	104	427	280	1155	×	×	×	×	×	×
	5·0	14	0	49	*	188	930	×	×	×	×	×	×
Sodium lauryl sulphate ..	0·5	19	63	69	364	105	452	130	565	161	678	198	854
	1·0	20	0	46	*	96	264	118	327	189	754	228	1005
	2·0	24	0	85	352	222	302	287	1357	389	2010	447	2764
	5·0	24	*	37	*	262	603	515	2111	×	×	×	×

* Pseudoplastic.
× Traces uninterpretable.

Sherman (1959) proposed that the emulsifier concentration was related to the viscosity and phase volume by an equation of the general form:

$$\ln \frac{\eta}{\eta_0} = a\,C\,\phi + b$$

where η and η_0 are the viscosities of the emulsion and the continuous phase respectively, ϕ the phase volume ratio, a and b are constants and C is the concentration of emulsifier. We applied this equation to our data for all emulsions containing oleyl alcohol and lauryl alcohol with non-ionic surfactants but did not obtain a linear plot for ln η/η_0 or η/η_0 against C for a constant value of ϕ. This suggested that the relation between the rheological properties and the concentration of water-soluble component was more complex than has been postulated by Sherman. In a later paper he modified the equation to take into account the globule size distribution (Sherman, 1963). For oil-in-water emulsions he found that increasing C caused greater heterogeneity of size distribution and the

formation of multiple phase globules, neither of which have been observed in our systems.

Our results for emulsions containing cetostearyl alcohol and an extended range of water-soluble component concentrations showed maxima in both apparent viscosity (Fig. 1) and static yield value (Fig. 2). These maxima became progressively less marked as the concentration of cetostearyl alcohol was reduced and were not obvious at 0·75 and 0·25% w/w where the products were thin milky fluids (Table 3). These findings

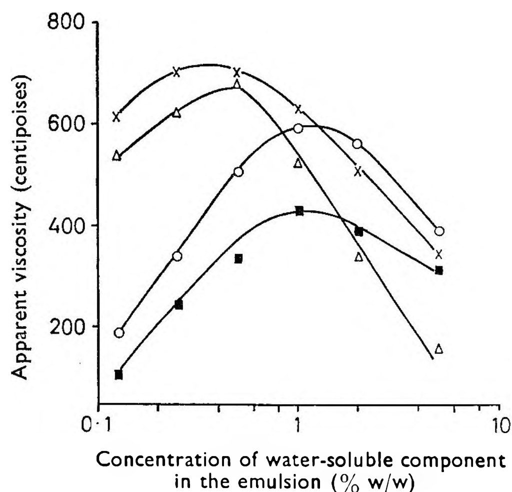

FIG. 1. Effect of water-soluble component concentration (%w/w) on apparent viscosity of emulsions containing 7%w/w cetostearyl alcohol. ■, Sorbester Q12. ○, Cetomacrogol 1000. △, Sodium lauryl sulphate. ×, Cetrimide.

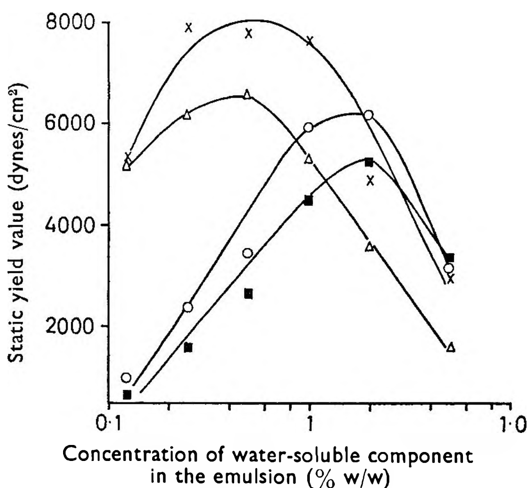

FIG. 2. Effect of water-soluble component concentration (%w/w) on static yield value of emulsions containing 7%w/w cetostearyl alcohol. ■, Sorbester Q12. ○, Cetomacrogol 1000. △, Sodium lauryl sulphate. ×, Cetrimide.

TABLE 3. EFFECT OF WATER-SOLUBLE COMPONENT CONCENTRATION ON APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF EMULSIONS CONTAINING CETOSTEARYL ALCOHOL

Water-soluble component		Cetostearyl alcohol concentration (% w/w)											
		0·25		0·75		1·5		2·5		4·0		7·0	
	% w/w	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV
Cetomacrogol 1000..	0·125	11	0	13	0	60	251	107	553	140	804	188	967
	0·25	10	0	12	0	55	226	156	892	248	1532	341	2362
	0·5	13	0	19	0	61	214	141	980	284	1508	506	3424
	1·0	14	0	15	0	42	*	149	980	340	2713	594	5905
	2·0	15	0	15	0	41	*	82	226	203	1489	562	6159
	5·0	33	0	36	0	63	*	94	151	141	276	393	3015
Sorbester Q12	0·125	10	0	12	0	47	201	58	301	84	502	107	603
	0·25	9	0	11	0	45	226	98	628	130	779	244	1558
	0·5	12	0	13	0	29	126	94	792	176	1407	336	2638
	1·0	8	0	7	0	23	*	50	402	172	1507	430	4522
	2·0	12	0	13	0	25	*	56	427	151	1307	392	5025
	5·0	15	0	19	0	39	*	54	364	117	879	315	3341
Cetrimide ..	0·125	10	0	11	0	44	125	76	377	206	1557	612	5339
	0·25	10	0	10	0	44	138	98	653	250	2211	701	†7915
	0·5	11	0	15	0	46	251	130	1005	269	2412	701	†7789
	1·0	13	0	18	0	29	75	92	704	182	1658	630	†7664
	2·0	18	0	21	0	28	126	50	452	151	1155	508	4900
	5·0	16	0	20	0	28	0	44	101	87	126	343	2952
Sodium lauryl sulphate ..	0·125	11	0	10	0	44	126	87	490	153	1206	534	5151
	0·25	12	0	13	0	35	138	139	942	225	1708	617	†6156
	0·5	12	0	18	0	52	251	120	936	269	1885	676	†6533
	1·0	15	0	22	0	32	57	117	949	172	1382	517	†5025
	2·0	26	0	32	0	21	25	50	402	101	817	343	3518
	5·0	13	0	38	0	33	0	37	0	45	0	154	1055

* Pseudoplastic.
† Mean static yield value.

are not in agreement with those of Axon (1956) who reported that the plastic viscosity of emulsions containing cetyl alcohol was directly proportional to the concentration of sodium lauryl sulphate between 0·2

TABLE 4. EFFECT OF WATER-SOLUBLE COMPONENT CONCENTRATION ON APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF GELS CONTAINING 10% W/W OIL-SOLUBLE COMPONENT

Oil-soluble component	Water-soluble component	Water-soluble component concentration (% w/w)									
		0·5		1·0		2·0		4·0		10	
		η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV
Oleyl alcohol	Cetomacrogol 1000	2	0	2	0	2	0	3	0	6	0
	Sorbester Q12	2	0	2	0	2	0	2	0	4	0
	Cetrimide	2	0	2	0	2	0	4	0	169	1859
	Sodium lauryl sulphate	2	0	3	0	5	0	18	0	48	*
Lauryl alcohol ..	Cetomacrogol 1000	20	370	16	*	48	440	142	2136	27	352
	Sorbester Q12	17	339	26	577	57	1658	197	2412	284	1758
	Cetrimide	162	2763	×	×	×	×	×	×	×	×
	Sodium lauryl sulphate	58	704	71	999	83	1162	167	1557	140	741
Cetostearyl alcohol ..	Cetomacrogol 1000	50	1319	102	3019	162	3731	261	4699	55	327
	Sorbester Q12	34	942	165	1187	220	1922	152	1093	29	936
	Cetrimide	118	1922	144	2355	177	2462	72	1721	44	377
	Sodium lauryl sulphate	107	1972	167	2915	175	2588	60	1212	30	150

* Pseudoplastic.
× Traces very irregular.

and 1·0% w/w. His emulsions had a lower phase weight ratio than ours and were subjected to mixing and heating processes after homogenization.

Caution is needed in directly comparing emulsion and gel data, since the exact amount of cetostearyl alcohol transferred to the aqueous phase is not known. However, it may be noted that gels formed by the dispersion of 10% w/w alcohol in varying concentrations of surfactant showed a maximum value of static yield value and apparent viscosity (Table 4). The precise position of the maxima for the latter cannot be

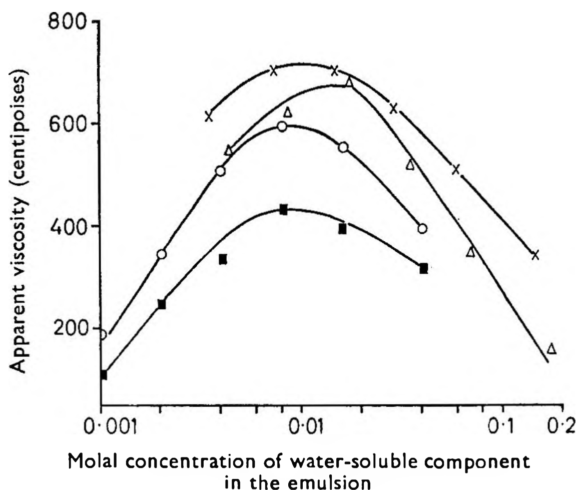

FIG. 3. Effect of water-soluble component concentration (molal) on apparent viscosity of emulsions containing 7%w/w cetostearyl alcohol. ■ Sorbester Q12. ○ Cetomacrogol 1000. △ Sodium lauryl sulphate. × Cetrimide.

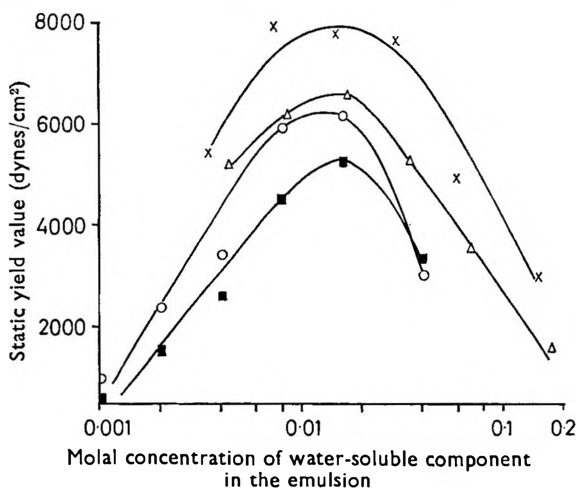

FIG. 4. Effect of water-soluble component concentration (molal) on static yield value of emulsions containing 7%w/w cetostearyl alcohol. ■, Sorbester Q12. ○, Cetomacrogol 1000. △ Sodium lauryl sulphate. × Cetrimide.

accurately assessed since inspection of the rheograms showed that some samples had not reached an equilibrium value of apparent viscosity at 100 rev/min and 600 sec sweep time.

The gels formed in the external phase of an emulsion by the interaction of cetostearyl alcohol and water-soluble component undoubtedly contain "structure" elements similar in function, but not necessarily in physical form, to those described by Barry & Shotton (1967) who examined aqueous gel systems containing only cetyl alcohol and sodium lauryl sulphate. The composition, number and relative proportion of such elements would presumably alter with the concentration of surfactant. At higher concentrations the amount of cetostearyl alcohol available for the production of "structure" elements may be reduced by solubilization and by complex formation similar to that described by Epstein, Wilson & others (1954).

The values for rheological parameters were generally higher for emulsions prepared with oleyl or lauryl alcohol and an ionic surfactant than with non-ionics. This effect was also apparent with cetostearyl alcohol. The lateral separation of the maxima with respect to percentage composition (Figs 1 and 2) largely disappeared with molal plots (Figs 3 and 4). The percentage concentrations at which the maxima occurred

TABLE 5. EFFECT OF CONCENTRATION OF SOLUMIN FX170SD AND TEXOFOR FX170 ON APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF EMULSIONS CONTAINING OLEYL, LAURYL OR CETOSTEARYL ALCOHOL

Water-soluble component		Oleyl alcohol concentration (% w/w)											
		1		2		4		6		8		10	
	% w/w	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV
Solumin FX170SD	0·5	11	0	14	0	17	0	19	0	26	0	35	0
	1·0	13	0	14	0	17	0	23	0	29	0	37	0
	2·0	13	0	18	0	31	0	30	0	37	*	49	*
	5·0	34	*	37	*	47	*	59	*	66	*	86	*
Texofor FX170	0·5	16	0	17	0	20	0	25	0	30	0	34	0
	1·0	14	0	16	0	19	0	25	0	32	0	37	0
	2·0	9	0	12	0	19	0	26	0	32	*	41	*
	5·0	34	*	38	*	47	*	53	*	68	*	81	*
		Lauryl alcohol concentration (% w/w)											
Solumin FX170SD	0·5	13	0	14	0	17	0	20	0	29	0	42	0
	1·0	14	0	16	0	17	0	22	0	29	0	40	*
	2·0	21	0	22	0	27	0	28	0	33	0	43	*
	5·0	33	*	38	*	42	*	54	*	70	*	82	*
Texofor FX170	0·5	16	0	13	0	10	0	14	0	16	0	30	0
	1·0	16	0	17	0	20	0	26	0	31	0	36	0
	2·0	10	0	11	0	23	0	27	0	34	*	42	*
	5·0	34	*	38	*	44	*	48	*	61	*	69	*
		Cetostearyl alcohol concentration (% w/w)											
		0·25		0·75		1·5		2·5		4·0		7·0	
Solumin FX170SD	0·5	11	0	13	0	–	–	–	–	–	–	–	–
	1·0	14	0	17	0	69	*	–	–	–	–	–	–
	2·0	27	0	22	0	25	*	–	–	–	–	–	–
	5·0	23	*	33	*	40	*	48	*	361	*	300	2362
Texofor FX170	0·5	13	0	14	0	16	0	49	389	–	–	–	–
	1·0	15	0	15	0	20	0	29	176	–	–	–	–
	2·0	15	0	17	0	22	*	29	101	147	†1156	229	1508
	5·0	31	*	37	*	37	*	45	251	122	603	172	1432

* Pseudoplastic.
† Mean static yield value.
– Emulsions contained wax-like particles and traces were very irregular.

TABLE 6. EFFECT OF WATER-SOLUBLE COMPONENT CONCENTRATION ON APPARENT VISCOSITIES (η_{100} IN CENTIPOISES) AND STATIC YIELD VALUES (SYV IN DYNES/CM^2) OF GELS CONTAINING 10% W/W OIL-SOLUBLE COMPONENT

Oil-soluble component	Water-soluble component	Water-soluble component concentration (% w/w)							
		1		2		4		10	
		η_{100}	SYV	η_{100}	SYV	η_{100}	SYV	η_{100}	SYV
Oleyl alcohol	Solumin FX170SD	1	0	1	0	2	0	4	0
	Texofor FX170	2	0	2	0	3	0	4	0
Lauryl alcohol ..	Solumin FX170SD	1	0	1	0	2	0	4	0
	Texofor FX170	2	0	3	0	4	0	32	*
Cetostearyl alcohol ..	Solumin FX170SD	Physical dispersions							
	Texofor FX170	Physical dispersions							

* Pseudoplastic.

would therefore appear to be dependent on molecular weight. In a preliminary attempt to elucidate whether the absolute magnitude of the maxima were dependent on molecular weight or charge we have investigated emulsions containing Solumin FX170SD (molecular weight about 1100-anionic), the corresponding non-ionic material Texofor FX170 and Texofor N4, a non-ionic material of low molecular weight (about 300).

The results of these experiments may be seen in Tables 5 and 6. It was found extremely difficult to prepare and test emulsions made with Texofor N4 and all three oil-soluble components, probably due to its solubility (about 2% w/w) in the oil phase. With oleyl and lauryl alcohol, Solumin FX170SD and Texofor FX170 both behaved like the high molecular weight non-ionic surfactants. The emulsions containing low concentrations of cetostearyl alcohol were thin stable fluids but all other members of the series contained wax-like particles which at low concentrations of water-soluble component gave irregular rheograms. Similarly, attempts to form gels produced thin structureless physical dispersions of the alcohol.

The data for emulsions containing Solumin FX170SD and Texofor FX170 with oleyl and lauryl alcohol suggest that molecular weight and probably shape were more important than charge. Results with cetostearyl alcohol yielded no further information in this respect. However, they do support the conclusion, noted previously, that a viscous emulsion will be formed only where the oil-soluble component migrates and interacts to give a gel in the continuous phase.

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The effect of electrolytes on rigidity and diffusion in gelatin–glycerin gels

J. R. NIXON, P. P. GEORGAKOPOULOS* AND J. E. CARLESS

The effect of electrolytes on the rigidity of and diffusion of dye from gelatin–glycerin gels has been examined. Cations, which might be expected to reduce the rigidity in the ratio of their valency, produced a fall in rigidity in the ratio Na^+, 1 : Ca^{++}, 1·25 : Al^{+++}, 1·57. This approximate halving of the effect is attributed to interference by the chloride ion. The rigidity was proportional to the square root of the ionic concentration except with sodium ferrocyanide where no simple relation held. A simple expression $G = G_0 (1 \pm \sqrt{I})$ was found for the rigidity, the sign depending whether anion or cation was considered. The effect of anions and cations on diffusion was as varied as their effect on rigidity. The addition of chlorides increased the diffusion coefficient in the order Al > Ca > Na. Both multivalent anions examined produced a decrease in diffusion coefficient; the sulphate being less effective than the ferrocyanide.

ELECTROLYTES have long been known to affect the rigidity of gelatin gels, even in the small amounts found in the ash of commercial material. Northrop & Kunitz (1926) and Bungenberg de Jong & Henneman (1932) reported a general lowering of rigidity at low concentrations of gelatin, whilst Narayanamurti & Gupta (1958) found the reverse. Cumper & Alexander (1952) found the rigidity to be decreased proportionately to the square root of the ionic strength.

Previously, Nixon, Georgakopoulos & Carless (1966, 1967) investigated factors controlling the rigidity and diffusion from gelatin–glycerin gels. This work has been extended to the effect of added electrolyte on rigidity and diffusion.

Experimental

MATERIALS

Gelatins. The characteristics have been given by Nixon & others (1966). *Glycerin* was Analar grade and *methylene blue* was of B.P. quality. *Electrolytes* were Analar grade and the *purified water* was once distilled from an all glass still (pH 5·2, specific conductivity 5 mhos cm^{-1}).

METHODS

The preparation of the gels, measurement of rigidity and the method of measuring the diffusion coefficient were as reported by Nixon & others (1966, 1967).

Results and discussion

The rigidity of gelatin–glycerin gels containing sodium chloride, calcium chloride, aluminium chloride, sodium sulphate and sodium ferrocyanide has been examined.

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An increase in the cation concentration caused a decrease in the rigidity of the gels at the gelatin–glycerin ratios studied (Figs 1 and 2). The rigidity could be expressed by the equation

$$G = G_0 (1 - K \sqrt{I}) \qquad \text{.. .. ..} \quad 1$$

where G_0 = rigidity in absence of electrolyte; I = ionic strength; K = constant (slope $/G_0$). This type of relation is similar to that reported by Cumper & Alexander (1952) but the numerical values of the constants are much smaller because of the effect of glycerin on the rigidity which is

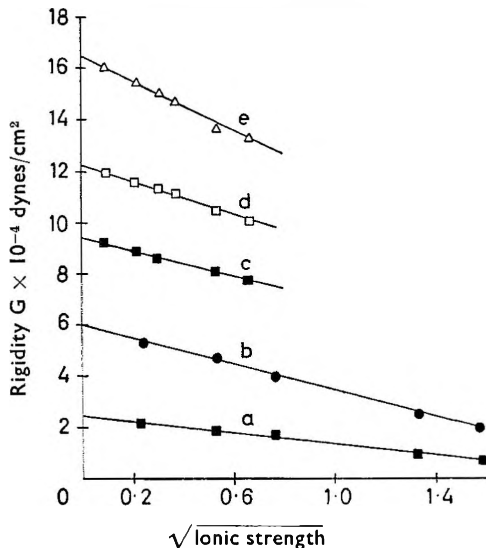


FIG. 1. Rigidity modulus as a function of the square root of the ionic strength of electrolyte. Gelatin Bloom strength: ● 99, ■ 154, □ 200, △ 250. % w/w gelatin–glycerin ratios: (a) = 5:20, (b, c, d, e) = 15:20. Electrolyte (a, b) = $AlCl_3$, (c, d, e) = NaCl. Temp. 25° ± 0·1°. Method of measurement: "Rigidometer".

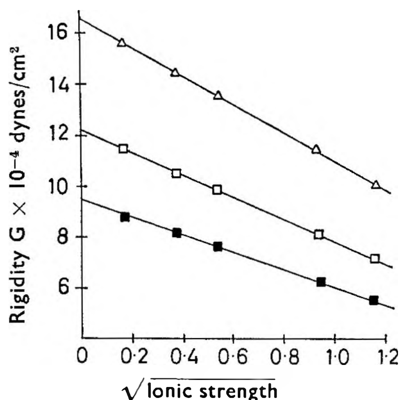


FIG. 2. Rigidity modulus as a function of the square root of the ionic strength of calcium chloride. Gelatin Bloom strength: ■ 154, □ 200, △ 250. % w/w gelatin–glycerin ratio: 15:20. Temp. 25° ± 0·1°. Method of measurement: "Rigidometer".

increased without a corresponding increase in the number of interchain linkages (Nixon & others, 1966). It was also observed that the addition of aluminium chloride caused shrinkage of the gels and that gelatin gels prepared from low Bloom number material exhibited viscoelastic properties (Fig. 3).

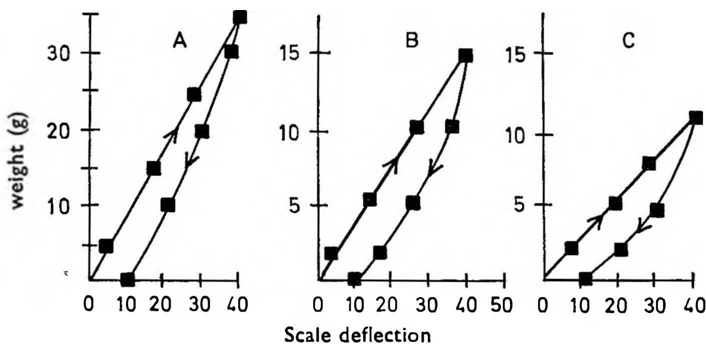


FIG. 3. Plastic behaviour of gelatin–glycerin gels in the presence of aluminium chloride. Gelatin Bloom strength: 154. % w/w gelatin–glycerin ratio: 10:20. $AlCl_3$: (A) 0·01M, (B) 0·15M, (C) 0·4M. Temp. 25° ± 0·1°. Method of measurement: "F.I.R.A. gel tester".

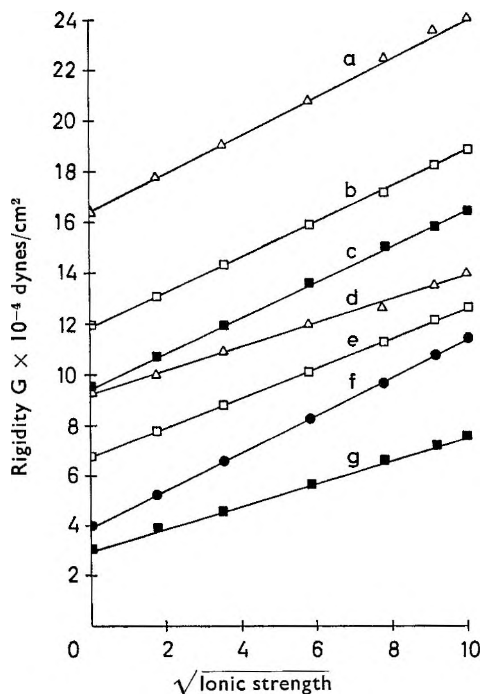
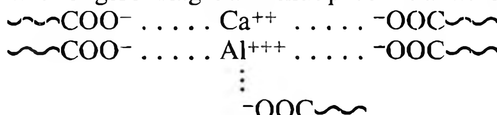


FIG. 4. Rigidity modulus as a function of the square root of the ionic strength of sodium sulphate. Gelatin Bloom strength: ● 99, ■ 154, □ 200, △ 250. % w/w gelatin–glycerin ratio: (a, b, c, f) = 15:20, (d, e, g) = 10:20. Temp. 25° ± 0·1°. Method of measurement: "Rigidometer".

The effect of cation valency on rigidity may be modified by the tendency of the chloride anion to reduce the rigidity by peptizing the gel linkages. The relative decrease produced by equimolar salt concentrations in gels with the same gelatin–glycerin ratio was approximately constant, but the absolute decrease was more pronounced with the chlorides of higher valency.

The metal cation opposed the reduction in rigidity produced by the chloride ion. Calcium and aluminium ions act through salt bridges to increase the number of gel linkages and thus produce a more rigid structure:



Because sodium is monovalent the sum total effect is that of the chloride ion. Using equation 1 it was possible to calculate the relative fall in rigidity produced by equi-ionic concentrations of the three salts.

		$NaCl$	:	$CaCl_2$	:	$AlCl_3$
Calculated	..	1	:	1·25	:	1·57
Expected	..	1	:	2	:	3

It will be seen that the overall effect of the calcium and aluminium salts

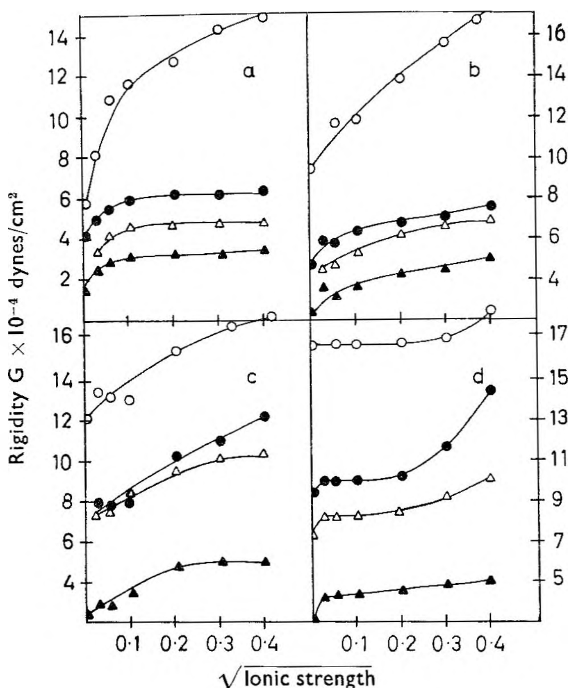


FIG. 5. Rigidity modulus as a function of the square root of the ionic strength of sodium ferrocyanide. Gelatin Bloom strength (a) 99, (b) 154, (c) 200, (d) 250. % w/w gelatin–glycerin ratio: ○ 15:20, ● 10:20, △ 8:20, ▲ 5:20. Temp. 25° ± 0·1°. Method of measurement: "Rigidometer".

is only approximately half that expected from their valency, indicating clearly the opposing effects of the anion and cation.

In contrast to the decrease in rigidity found with sodium chloride, the presence of multivalent anions such as sulphate and ferrocyanide produced an increase in rigidity (Figs 4 and 5). This increase was relatively greatest at low anion concentrations whilst at high concentration of gelatin the gel produced was almost rigid. A simple linear relation was found for sulphate ion, the only difference from equation 1 being a change of sign.

$$G = G_0 (1 + K \sqrt{SO_4^{--}}) \quad .. \quad .. \quad .. \quad 2$$

No such simple relation could be established for sodium ferrocyanide. Whilst the rigidity increased steeply with increasing low concentrations of ferrocyanide a constant value was approached at higher concentrations (Fig. 5a–c). The concentration of gelatin affected the behaviour of the system to this anion. With gelatins of up to 200 Bloom jelly strength, the increase in rigidity the anion produced with 15% gelatin gels was far more pronounced than that at lower gelatin concentrations. The 250 Bloom gelatin showed a completely different ferrocyanide effect. With these gels a concentration of up to 0·2M ferrocyanide produced only a slight increase in rigidity, but at anion concentrations above this the rigidity increased steeply (Fig. 5d).

Because of the non-interference of the monovalent sodium ion the increased rigidity produced by sodium sulphate and ferrocyanide must be caused by the multivalent anion. As with the multivalent cations calcium

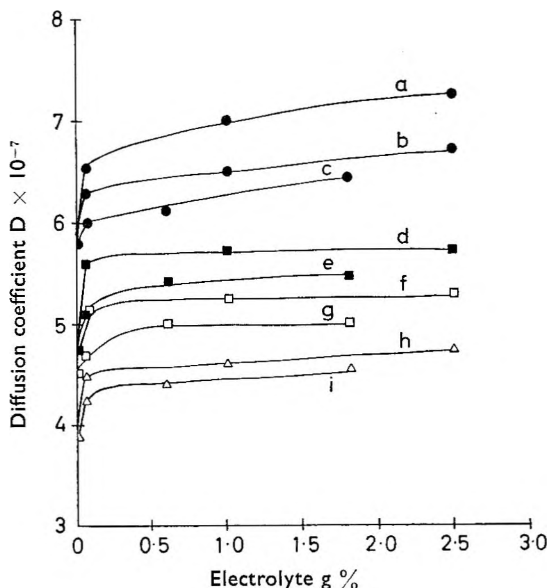


FIG. 6. The effect of anion concentration on the diffusion coefficient of methylene blue from gelatin–glycerin gels. Gelatin Bloom strength: ● 99, ■ 154, □ 200, △ 250. % w/w gelatin–glycerin ratio 10:20. Methylene blue 9·5 mg %. Electrolyte: (a) $AlCl_3$, (b, d, f, h) $CaCl_2$, (c, e, g, i) NaCl. Temp. 25° ± 0·1°.

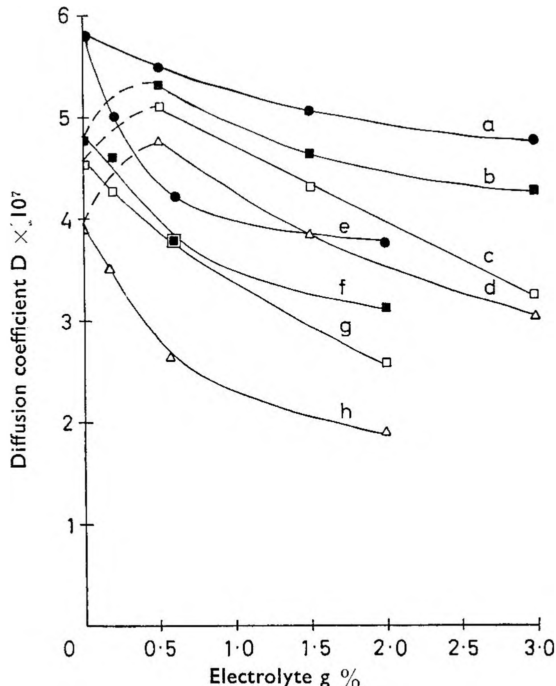


FIG. 7. The effect of cation concentration on the diffusion coefficient of methylene blue from gelatin–glycerin gels. Gelatin Bloom strength: ● 99, ■ 154, □ 200, △ 250. % w/w gelatin–glycerin ratio: 10:20. Methylene blue 9·5 mg %. Electrolyte: (a, b, c, d) Na_2SO_4; (e, f, g, h) $Na_4[Fe(CN)_6]$. Temp. 25° ± 0·1°.

or aluminium, these anions can form salt bridges between the gelatin chains and thus increase the rigidity.

These salt linkages are non-directional and therefore the existence of a bond does not necessarily imply that it will contribute to the shear rigidity. Only those bonds opposing the chain in the particular direction in which the force was applied would be effective.

The difference in the effect of cations and anions on the diffusion of methylene blue from the gels (Figs 6 and 7) was as sharp as that on the rigidity. The addition of the chlorides increased the diffusion coefficient in the order $AlCl_3 > CaCl_2 > NaCl$. The multivalent anions produced a decrease in diffusion coefficient, the sulphate being less effective than the ferrocyanide. Whilst the effect of the electrolytes was more pronounced with high Bloom number gelatins, no simple relation could be derived for diffusion coefficient and either salt concentrations or ionic strength.

The change in diffusion rate can be linked with changes in the gel microstructure. The chlorides increase the pore size by peptizing certain of the gel linkages and allowing fewer points of contact along the gelatin chain. Conversely the pore size of gels will tend to be decreased by the presence of calcium and aluminium ions. It is the overall balance between these two conflicting factors which results in the decrease in rigidity

accompanied by an increased pore size, and consequently an increased diffusion coefficient. The multivalent cations decrease the pore size by providing additional linkages between adjacent chains so that not only does the rigidity increase but the diffusion coefficient falls. The gelatins of high Bloom strength exhibited these effects in the most pronounced form because the long-chain length materials present allow more linkages per molecular unit.

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Gelatin coacervate microcapsules containing sulphamerazine: their preparation and the *in vitro* release of the drug

J. R. NIXON, SALEH A. H. KHALIL* AND J. E. CARLESS

An improved method is described for the preparation of gelatin coacervate microcapsules containing sulphamerazine as a fine deflocculated powder. The factors which control both the coacervation step and the recovery of the microcapsules are discussed. The *in vitro* release of sulphamerazine from microcapsules of different wall thickness which had been hardened by formaldehyde under different conditions has been studied. The method of preparation gave a high percentage of encapsulated material in comparison with other recovery techniques.

IN recent years investigations have been made into the ability of coacervate droplets to surround small particles dispersed in the coacervating system. Applications of this technique have been found in the preparation of carbonless copying paper (Green 1957; Green & Schleicher 1956a,b) and the microencapsulation of a number of pharmaceuticals (Phares & Sperandio, 1964; Luzzi & Gerraughty, 1964, 1967a, b). Although little difficulty appears to have been found in the coacervation step the recovery techniques reported indicate the difficulty encountered in producing a fine powder. In the patent literature spray-drying or freeze-drying of the microcapsule suspension is suggested or alternatively comminution of the hard cake produced after filtering the gelled microcapsules (Green & Schleicher, 1956a,b). None of these recovery techniques appears to have been critically evaluated. The methods of Phares & Sperandio (1964) and Luzzi & Gerraughty (1964) might be expected to produce a significant proportion of "free" material due to mechanical rupture during either comminution of the cake or passage of the gelled mass through a sieve.

We have examined the preparation of sulphamerazine microcapsules using the simple coacervating systems: gelatin–water–ethanol and gelatin–water–sodium sulphate. A fine powder essentially without "free" sulphamerazine was produced without the need of mechanical treatment. The *in vitro* release of the drug from these microcapsules was also examined.

Experimental

MATERIALS

Two samples of *gelatin* were used having the characteristics given in Table 1. The *gelatins* were dried in thin layers at 110° for 12 hr and stored in air-tight containers. *Absolute ethanol* and 20% w/w *sodium sulphate* (A.R. grade) solution were used as the coacervating agents. *Sulphamerazine* was of B.P. (1953) quality. Most of the particles were between 2 and 5 μ diameter under the microscope.

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* The work forms part of a thesis by S. A. H. Khalil submitted for the degree of Ph.D. in the University of London.

TABLE 1. CHARACTERISTICS OF GELATIN SAMPLES

Sample	Type	Source	Bloom No.	pH	Viscosity (Cps, 6·67%)	I.E.P.	% Ash
A	Lime-pretreated	Hide	240	6·6	8·1 (40°)	5·2	1·20
B	Acid-processed	Pigskin	252	4·7	5·5 (60°)	9·2	1·11

PREPARATION OF MICROCAPSULES

As a preliminary to microencapsulation, phase boundary triangular diagrams were prepared for each system (Nixon, Khalil & Carless, 1966), and suitable compositions within the coacervate region chosen. The general outline of the coating procedure followed that given in British Patent 751,600 (1956). The material to be encapsulated was dispersed in ethanol or 20% w/w sodium sulphate solution and added to the iso-electric gelatin solution. The mixture was continuously agitated by a variable speed paddle stirrer and maintained at 40°. Further coacervating agent was added over 30–40 min and the formation of coacervate coated particles was verified microscopically.

RECOVERY TECHNIQUES FOR MICROCAPSULES

(*a*) Depending upon which coacervating agent was used to prepare the microcapsules, the product was poured into five times its volume of either 30% w/w aqueous ethanol or 7% w/w sodium sulphate solution at 5°. This resulted in the gelling of the hitherto liquid shell of the microcapsules. The dispersion was continuously agitated for 30 min. After centrifugation at 500 rev/min for 5 min the mother liquor was decanted and the product washed with isopropanol at 5° for 30 min. It was then possible to filter the fine microcapsules and after washing with ethanol they were dried at room temperature. The coacervate shell of the recovered microcapsules was hardened by suspending 1·0 g of the microcapsules in 10 ml of formalin–isopropanol mixture (1:4 v/v). The formalization time ranged from 15 min to 3 days and the formalized microcapsules were filtered, washed with water and dried at 70° for 12 hr. This method was the most satisfactory, the product being recovered as a deflocculated fine powder. The porosity of the coacervate walls, particularly in sodium sulphate coacervated systems, depends upon the rate of gelling of the coacervate material. For this reason the experimental procedure was rigidly controlled.

(*b*) The coacervate microcapsules were gelled by reducing the temperature to 5°. They were not treated with isopropanol. Formaldehyde was added to the gelled microcapsules to produce a final concentration of 10% w/v formaldehyde. The mixture was stirred at 5–10° for 30 min, filtered, washed with water and dried at 70° for 12 hr. This method produced a cake which had to be further comminuted in a mortar or mill and the product fractionated.

(*c*) The prepared microcapsules were not gelled or treated with formaldehyde but spray-dried using a Portable Spray Drying Minor Unit (Niro Atomizer Ltd.). The feed rate of 5 ml/min was controlled by a pulsating pump. The operating conditions for spray drying are shown in Table 2.

TABLE 2. OPERATING CONDITIONS FOR SPRAY DRYING THE FORMALIZED SUSPENSION OF COACERVATE MICROCAPSULES CONTAINING SULPHAMERAZINE

Experiment*	Compressed air (kg/cm^2)	Heat control	Temp. ranges, °C		Cooling Ports
			Inlet	Outlet	
A	2	6	220–230	120–130	Fully open
B	3	4	130–140	75–80	Fully open
C	4	3	110–120	65–70	Partially open
D	5	2	100–110	55–60	Partially open
E	2	1	100	45–50	Partially open
F	4	2	100–110	50–55	Fully open

Feed rate in all experiments: 5 ml/min.
* Mean values from 5 replicates.

ASSAY OF SULPHAMERAZINE IN VARIOUS PRODUCTS

Total sulphamerazine. The formogelatin coat was decomposed with 50% v/v hydrochloric acid at 60° for 5–20 min (Bogue, 1922). A concentration of 0·1 g of the recovered powder per 40 ml of acid was used. Five ml of the clear solution produced was then assayed by the method of Bratton, Marshall & others (1939), using a photoelectric absorptiometer (Evans Electroselenium Ltd.) and a No. 605 filter. The mean of five determinations was calculated.

"Free" sulphamerazine. The product, 0·1 g, was shaken with 25 ml of acetone at room temperature to dissolve any unencapsulated sulphamerazine. After centrifuging, an aliquot was assayed for sulphamerazine content as previously.

MEASUREMENT OF *in vitro* RELEASE FROM MICROCAPSULES

A rotating bottle apparatus similar to that of Souder & Ellenbogen (1958) was used. About 0·9 g of the coacervate microcapsules, accurately weighed, was placed in a 90 ml screw-capped bottle containing 40 ml of the dissolution medium (acid pepsin solution B.P. or alkaline pancreatin solution B.P.). The bottles were rotated at 40 rev/min at 37° ± 0·05°. After various time intervals the dispersions were centrifuged for 1 min at 500 rev/min and an aliquot of the clear solution assayed for sulphamerazine released.

$$\text{\% sulphamerazine released} = \frac{\text{mg sulphamerazine released/g microcapsule}}{\text{mg total sulphamerazine/g microcapsule}} \times 100\%$$

SIZE ANALYSIS OF MICROCAPSULES

The size of the microcapsules was determined by direct measurement of the diameter of the magnified microcapsules projected on the screen of a Projectina microscope. The results were expressed as number percentage cumulative curves.

Results

PREPARATION AND RECOVERY OF THE MICROCAPSULES

The effect of gelatin type and the coated material on the coacervation step was studied. The results are shown in Tables 3 and 4. Factors

TABLE 3. EFFECT OF GELATIN TYPE ON MICROENCAPSULATION OF SULPHAMERAZINE

Gelatin type	pH of the gelatin solution	% w/w coacervating agent added to produce phase change		Microencapsulation	
		Ethanol	Sodium sulphate	Ethanol system	Sodium sulphate system
Lime-pretreated	6·6*	45·9	8·7	+	+
Acid-processed	4·7*	62·4	8·0	− (Floccules)	+
	6·1	60·1	8·6	− (Floccules)	+
	7·0	57·4	8·9	+	+
	8·4	55·2	9·2	+	+

* pH of the gelatin solution without adjustment.
Initial gelatin concentration: 8% w/w.

TABLE 4. EFFECT OF THE MATERIAL TO BE COATED ON THE OCCURRENCE OF COACERVATION. Minimum ethanol or sodium sulphate concentrations (% w/w in the total system) required to initiate coacervation

Material*	Gelatin–water–ethanol system			Gelatin–water–sodium sulphate system		
	Initial gelatin concentration (% w/w)					
	4	8	16	4	8	16
Blank (no material)	43·1	41·4	37·7	8·4	8·2	8·0
Sulphamerazine	43·6	41·8	36·1	8·4	8·1	7·9
Aspirin	54·2	49·5	44·2	8·0	7·7	7·3
Glass beads (53μ)	46·3	44·7	40·4	8·9	8·6	8·7
Phenolphthalein	68·7	63·4	58·5	9·3	9·2	9·0

* 10 g of the material dispersed in 100 g of the isoelectric gelatin solution maintained at 40° ± 0·1°.
Gelatin used: 240 Bloom, lime-pretreated sample.

which produced changes in pH or interaction with the gelatin molecule influenced the onset of coacervation and therefore microencapsulation. In this category was the alkalinity of the glass beads and the acidity due to aspirin, whilst with phenolphthalein the lyotropic effect of the phenolic group on the gelatin molecule (Gustavson, 1956) suppressed coacervation particularly in the ethanol system.

Changes due to gelatin type followed a similar pattern to that reported in our previous paper (Khalil, Nixon & Carless, 1968). With alkali-processed gelatin without pH adjustment it was possible to encapsulate sulphamerazine using either ethanol or sodium sulphate as the coacervating agent. The acid processed gelatin had to be adjusted to pH values in the vicinity of its isoelectric point before encapsulation was achieved using ethanol, but with sodium sulphate no adjustment was required within the pH range studied. This was in accordance with previous results (Khalil & others, 1968) where it was found that with ethanol systems, using alkali commercial gelatins, the effective range for coacervation was between pH values 4·6 and 9·2 and for acid processed gelatins 6·9–10·8. With sodium sulphate coacervates the effective pH range was found to be 2·1–10·5 irrespective of the gelatin type. The encapsulated material appeared to be evenly distributed throughout the coacervate droplets. These droplets tend to aggregate if the amount of coacervating

agent used to produce them was close to either of the boundaries of the coacervate region (Fig. 1). At the onset of coacervation only the higher molecular weight fractions of the gelatin coacervated and formed the mantle of the microcapsule. The lower molecular weight fractions remained in the equilibrium liquid and on gelling were deposited on the outside of the microcapsules which then tended to agglomerate on contact. This occurred with both coacervating systems. Near the three phase zone of the triangular diagram, the coacervate phase on analysis was found to be richer in gelatin and poorer in water whilst the equilibrium liquid contained negligible amounts of gelatin (Nixon & others, 1966). The highly viscous nature of the coacervate under these conditions enhanced the agglomeration of the coacervate droplets.

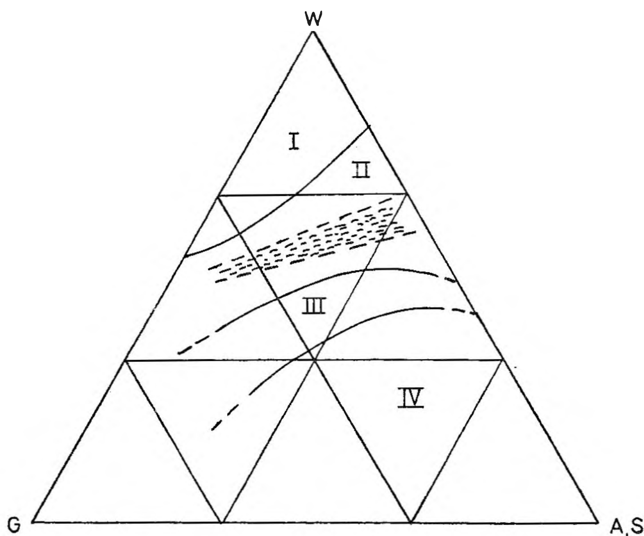

FIG. 1. Schematic representation of the optimum region for microencapsulation. I, clear solution. II, coacervate region (etched portion produces optimum conditions for microencapsulation). III, three phase region. IV, flocculate region.

The optimum conditions for the production of microcapsules is shown schematically in Fig. 1 and occurs in a region almost midway in the coacervation zone. In this region all the gelatin fractions had coacervated yet the viscosity had not appreciably increased.

Whilst the formation of a coacervate coat around a particle was easily accomplished from a study of the physical characteristics of the system, the recovery of the microcapsules in an acceptable form as a fine powder presented difficulties.

Filtration under varying conditions quickly led to blockage of the filter pores and production of a rubbery cake. Prior treatment with formalin did not produce any marked improvement. The cake produced after drying was comminuted and sieved to give three fractions. These were then assayed for "free" sulphamerazine (Table 5). The two coarser

fractions contained up to 25% w/w unencapsulated material whilst the fine fraction was almost completely in the "free" form. This adequately indicates that comminution would be accompanied by rupture of the coacervate shell depending on the extent of comminution. This result was also anticipated from the use of coacervate microcapsules in the preparation of carbonless copying paper.

TABLE 5. "FREE" SULPHAMERAZINE IN PRODUCTS RECOVERED BY VARIOUS METHODS*

Method of recovery	Average % of free sulphamarazine	
	Ethanol system	Sodium sulphate system
A Isopropanol method		
(a) Before acetone washing	7·1	4·3
(b) After acetone washing at 20° for 15 min	3·6	1·8
B Comminution of the filter cake		
(a) Fraction > 44 mesh	11·4	9·8
(b) Fraction >170 mesh	25·3	24·1
(c) Fraction <170 mesh	97·0	98·2
C Spray drying (all products obtained) ..	98·3	98·6

* All products obtained were prepared under the same conditions and the ratio of gelatin to sulphamerazine was 8·4. The products were formalized for 30 min.

Spray dried products needed no further comminution and consisted of fine free-flowing powder which passed through a 300 mesh sieve. The best conditions for operating the spray drier were D and F (Table 2). However, the spray dried products were entirely unacceptable as almost all the sulphamerazine was unencapsulated. Microscopic examination showed empty spherical particles 2–5 μ in diameter. This was much smaller than the size of the microcapsules in the feed suspension. The gelled microcapsules could probably not withstand the shearing force of the atomizer which ruptured the microcapsules. Spray drying, under far gentler conditions than here available, may be possible.

The only successful method of producing microcapsules as a dry deflocculated powder was by treating the centrifuged slurry with water-miscible aliphatic alcohols which possess the property of precipitating fibres from soluble collagens. Isopropanol was found to be superior to n-propanol or ethanol in that it gave a product with a negligible amount of agglomerates. The sedimentation time and the type of product

TABLE 6. COMPARATIVE EFFECTS OF ETHANOL, PROPANOL AND ISOPROPANOL ON SULPHAMERAZINE COACERVATE MICROCAPSULES*

The alcohol used	Time of complete sedimentation (min)	Sedimentation volume (ml)	Quality of the sediment
Ethanol	6	42	lumpy agglomerates
n-Propanol	10	31	coarse aggregates
Isopropanol	24	18	very fine particles

* 25 g of the centrifuged microcapsule slurry were separately treated with equal volume of the alcohol in a 100 ml measuring cylinder.

obtained after treatment are shown in Table 6. In this experiment 25 g of the centrifuged microcapsules slurry were treated with equal volumes of the alcohol in a 100 ml cylinder.

The size distribution of the microcapsules and the effect of the isopropanol treatment is shown in Fig. 2. This recovery technique produced the smallest quantity of "free" sulphamerazine. The small amount of "free" material is ascribed to the solubility of the sulphamerazine used in the system. This produces external contamination of the surface layers of the microcapsules. Washing with acetone reduced this surface contamination to less than 2%.

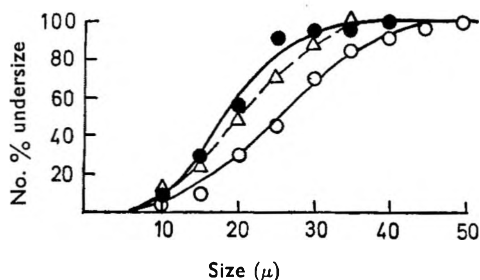

FIG. 2. Number size distribution of empty coacervate droplets, ●; freshly prepared microcapsules of sulphamerazine, ○; and microcapsules after hardening by isopropanol, △. System: gelatin–water–ethanol. % w/w gelatin/sulphamerazine ratio 8:4.

In vitro RELEASE OF SULPHAMERAZINE FROM COACERVATE MICROCAPSULES

This was examined under a number of conditions. A maximum of 6 hr of formalization was used, as longer periods allowed interaction between sulphamerazine and the diffused formaldehyde solution which resulted in the formation of a soluble complex which was leached out into the formalin–isopropanol mixture. At formalization times less than 6 hr no loss of sulphamerazine from the microcapsule could be detected.

The results of release experiments, Figs 3–6, showed that hardened microcapsules retarded the release of sulphamerazine into both acid pepsin and alkaline pancreatin solutions. The effect was more pronounced in the acid pepsin solution and depended on both the formalization time and the gelatin/sulphamerazine ratio. After 8 hr in this dissolution medium the formalized microcapsules were still intact, but in alkaline pancreatin solution they ruptured after various time intervals depending on their length of pretreatment with formalin-isopropanol mixture (Fig. 4). When compared with the effect in acid pepsin the thickness of the gelatin coat, as measured by the gelatin/sulphamerazine ratio, produced an insignificant effect on the release pattern in alkaline pancreatin solution (Fig. 5). It should also be noted that the release of the drug was faster from sodium sulphate coacervated microcapsules than from similar formulations using ethanol as the coacervating agent.

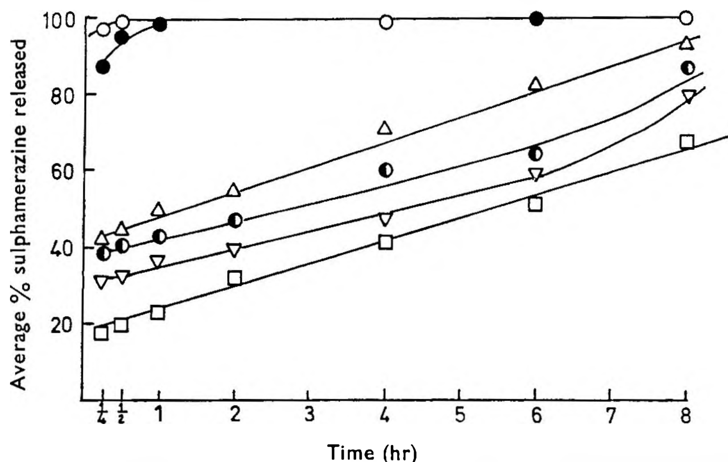

FIG. 3. *In vitro* release of sulphamerazine from ethanol coacervated microcapsules. % w/w gelatin/sulphamerazine ratio 8:4. Dissolution medium: acid pepsin solution B.P. Temperature 37° ± 0·05°. ○, Sulphamerazine crystals; ●, unformalized microcapsules; △, microcapsules formalized for 15 min; ◐, microcapsules formalized for 1 hr; ▽, microcapsules formalized for 3 hr; □, microcapsules formalized for 6 hr.

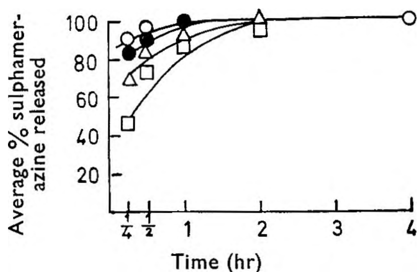

FIG. 4. *In vitro* release of sulphamerazine from ethanol coacervated microcapsules. % w/w gelatin/sulphamerazine ratio 8:4. Dissolution medium: alkaline pancreatin solution B.P. Temperature 37° ± 0·05°. ○, Sulphamerazine crystals; ●, microcapsules formalized for 15 min; △, microcapsules formalized for 1 hr; □, microcapsules formalized for 6 hr.

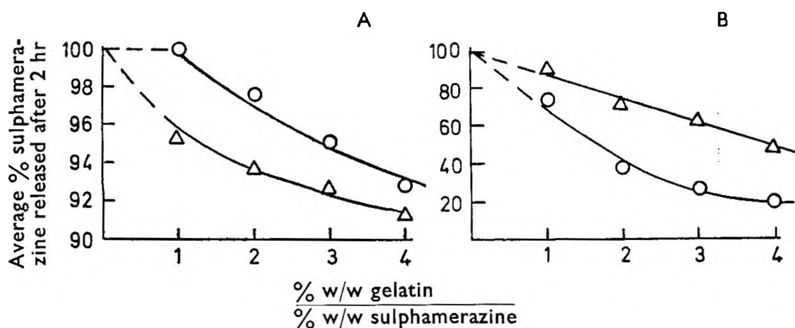

FIG. 5. Effect of thickness of microcapsule coat on the release of sulphamerazine. Dissolution medium A: alkaline pancreatin solution. B: acid pepsin solution B.P. B.P. Formalization time: 3 hr. Coacervating agent: △, sodium sulphate; ○, ethanol. Temp. 37° ± 0·05°.

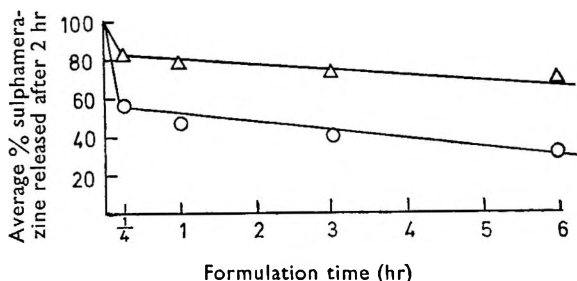

FIG. 6. Effect of formalization time on the release of sulphamerazine. % w/w gelatin/sulphamerazine ratio: 8:4. Dissolution medium: acid pepsin solution B.P. Coacervating agent: △, sodium sulphate, ○, ethanol. Temp. 37° ± 0·05°.

Discussion

Since no detailed studies have been reported on microencapsulation of pharmaceuticals by gelatin coacervation, it was necessary to investigate the various conditions under which a solid could be coated and recovered as a dry powder. Because only the coacervate phase shows the unique property of encapsulating solid materials it is important to study those factors which influence the location of the coacervate phase within the triangular diagram. Materials which produce changes in pH or interact with the gelatin could result in the displacement of the coacervate region inside the triangular diagram (Nixon & others, 1966; Khalil & others, 1968). Even within the coacervate region there was an optimum zone in which the coacervate droplets possessed a minimum aggregation potential and it was only within the zone that a deflocculated product was successfully prepared.

The size of the material to be coated did not limit the ability of the coacervate droplets to coat them and it was also found that encapsulation occurred whether the particles were dispersed in the gelatin solution before the coacervation step or added to an already coacervated system. This suggests that microencapsulation can occur by two mechanisms: (*a*) by the dispersed particles acting as seeding nuclei around which the coacervate droplets form; or (*b*) the droplets in the presence of dispersed particles may absorb them by an invagination mechanism.

The recovery of encapsulated material has been reported by a number of workers without any detailed evaluation of the proposed methods being attempted. Filtration appears to produce a rubbery mass which on drying yields a hard cake. The comminution of the cake (Phares & Sperandio 1964, Brit. Pat. 1956) or the passage of the rubbery mass through a sieve (Luzzi & Gerraughty, 1964) would affect the integrity of the coacervate coat. As seen from Table 5 a large proportion of the sulphamerazine was unencapsulated or in cracked microcapsules. This we anticipated, since the basic principle of the use of coacervate microcapsules in carbonless copying paper is that they will rupture under the mechanical pressure of writing or typing. The present method resulted in the complete encapsulation of the sulphamerazine provided adequate

coacervate material was present. As shown in Table 5, 7% by ethanol coacervation or 4% by sodium sulphate coacervation was either unencapsulated or present in broken capsules.

The simple procedure suggested in the present paper was based on hardening the coacervate shell by dehydration. Formalin was unsatisfactory due to its water content. The work of Bensusan (1960) showed that high concentrations of water-miscible aliphatic alcohols favoured precipitation of soluble collagens in the order: methanol > ethanol > n-propanol > isopropanol. Ethanol possessed too strong a dehydrating effect for the present purpose and resulted in shrinkage and agglomeration of the microcapsules. However, isopropanol, with its milder dehydrating effect, resulted in the production of a very fine free-flowing powder and in contrast to other methods of recovery, no rupturing of the coacervate coat occurred.

The *in vitro* release studies were only possible on the powders recovered by the isopropanol method. The coacervate coat retarded the release of the encapsulated sulphamerazine, the effect depending on the dissolution medium, time of formalization and thickness of the gelatin coat. It was found by Tanaka, Takino & Utsumi (1963) that formalized gelatin was less sensitive to hydrolysis by protease enzyme. This meant that by varying formalization time the rate of release could be controlled. Due to the reaction between formaldehyde and the primary amino-group of the sulphamerazine the formalization time was limited to 6 hr. Nevertheless the effect of formalization time is well shown in Fig. 3. After only 15 min in the acid pepsin, approximately 20% of the sulphonamide was released even when long periods of formalization were used. The degree of protection afforded against acid pepsin is much less than when a hard gelatin capsule is treated for enteric coating as the walls of the microcapsule are much thinner. Similarly the thickness of the coacervate coat effectively delayed the release, most probably by inhibiting the rate of diffusion of the dissolution medium into the microcapsules. The release of encapsulated material into alkaline pancreatin solution exhibited a totally different pattern from acid pepsin solution as the gelatin coat ruptured. The release from sodium sulphate-coacervated microcapsules was relatively higher than with similar ethanol coacervated products. The sodium sulphate not only hinders the hardening effect of the isopropanol (Bensusan & Hoyt, 1958) during the recovery technique, but also yields a highly porous coat under the conditions of the *in vitro* release experiments as the sodium sulphate dissolves from the microcapsule shell.

Whether the structure of the coacervate coat differs from that of a gel is still not certain in spite of the marked birefringence of the former (Bungenberg de Jong, 1938). Maier & Scheuerman (1960), from electron microscopy and diffusion studies, have found that coacervate films of cellulose nitrate of a graded porosity could be prepared by the correct choice of the solvent-non solvent system and drying conditions.

Diffusion through the coacervate coat may well occur through the intermolecular spaces of the coiled structure as well as through the tiny coacervate vacuoles which can be seen in the coacervate droplets.

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Aspects of the epimerization of certain tetracycline derivatives

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The epimerization of several tetracycline derivatives was examined at several pH values using varying conditions of temperature and buffer strength. Rate coefficients for the epimerization of tetracycline and demethylchlortetracycline are reported and factors associated with the epimerization of chlortetracycline and oxytetracycline are discussed. Under the conditions used calcium had no effect on the rate of epimerization and copper promoted degradation other than epimerization.

THE C-4 epimerization of tetracycline and its analogues was first described by Doerschuk, Bitler & McCormick (1955). The epimer differed significantly from the analogue of normal configuration in several of its properties, the most important being its *in vitro* antibiotic activity. This was found to be less than 5% of the activity of the normal analogue.

Conditions promoting and inhibiting epimerization have been described by McCormick, Fox & others (1957). They found that epimerization occurs in a variety of solvent systems within the pH range of approximately 2–6 and that the rate of epimerization is increased by certain buffering agents. Remmers, Sieger & Doerschuk (1963) found that this C-4 epimerization follows first-order reversible reaction kinetics.

An understanding of the epimerization process and the factors that influence it is important in the preparation of liquid pharmaceutical dosage forms since a loss in potency can result from spontaneous epimerization. Also there have been reports of kidney damage following the ingestion of degraded tetracycline capsules (Frimpter, Timpanelli & others, 1963) and 4-epitetracycline and 4-epianhydrotetracycline were identified among the degradation products. 4-Epianhydrotetracycline has been specifically cited as the agent causing the kidney damage (Benitz & Diermeier, 1964; Lowe & Tapp, 1966). Thus, the possibility of epimerization must be considered when evaluating the stability and potential toxicity of formulations of the tetracycline drugs. We have expanded earlier epimerization studies and examined the influence of calcium and copper on epimerization.

Experimental

REAGENTS

Samples of tetracycline hydrochloride, chlortetracycline hydrochloride and demethylchlortetracycline hydrochloride were supplied by Lederle Laboratories and oxytetracycline hydrochloride was donated by Chas. Pfizer and Company. Analytical reagent grade sodium acetate was used to make approximately 0·1M and 1M buffer solutions at pH values 4·0, 5·0 and 6·0, the solutions being adjusted to the appropriate pH with

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concentrated hydrochloric acid. The solutions containing calcium or copper salts were prepared using analytical reagent grade copper chloride ($CuCl_2.2H_2O$) and calcium chloride ($CaCl_2.2H_2O$). Approximately 0·1N sulphuric acid solutions were used for the spectrophotometric assays; these were made with a Hitachi Perkin-Elmer spectrophotometer.

PROCEDURE

Solutions of the appropriate buffer were prepared and adjusted to the required pH. The buffers were allowed to equilibrate at the desired temperature in 50·0 ml volumetric flasks wrapped in aluminium foil to shield the solutions from light. The tetracycline derivative was added to the buffer to give solutions that were approximately 6×10^{-4}M with respect to the tetracycline derivative. At intervals, samples were withdrawn, diluted with 0·1N sulphuric acid, and assayed spectrophotometrically using an absorbancy ratio analysis to be described. These studies were made at pH values of 4·0, 5·0 and 6·0 in both 0·1M and M acetate buffers at temperatures of 30, 37 and 50° which were maintained by a regulated water bath. When the influence of calcium or copper on the epimerization was examined, the concentration of the metal was varied in the initial solution to give molar ratios of metal : tetracycline ranging from 1 : 5 to 5 : 1.

Results and discussion

A modification of the absorbancy ratio assay of McCormick & others (1957) was used to follow the epimerization. This assay makes use of significant differences in the spectral curves of tetracyclines and their epimers at 254 and 267 mμ when 0·1N sulphuric acid is used.

Remmers & others (1963) modified this assay to observe the kinetics of tetracycline epimerization. This assay is useful for kinetic studies because the time between sampling and completion of the assay is short. Also, since 0·1N sulphuric acid is used, any complex between a tetracycline and a metal would be broken and therefore the free tetracyclines could be assayed without interference from metal chelates.

For the assay the mixture being analyzed must contain only a tetracycline and its corresponding epimer. Because of this, preliminary studies were made to determine its limitations. Solutions of tetracycline hydrochloride with pH values adjusted between the limits of pH 3·0 and 8·0 using sodium hydroxide or hydrochloric acid (buffers increase the rate of epimerization) were used. The absorbance of the solutions was measured at 254 and 267 mμ and curves made simultaneously, scanning from 380 to 220 mμ.

Appreciable degradation other than epimerization quickly took place at pH values above 6·0. At lower pH values, degradation other than epimerization was evident over a longer time, as seen by decreased absorbance in the 300–380 mμ region (Fig. 1).

To overcome this problem the original assay was modified. McCormick & others (1957) and Remmers & others (1963) observed epimerization to occur at a faster rate in the presence of buffers, the rate increasing with

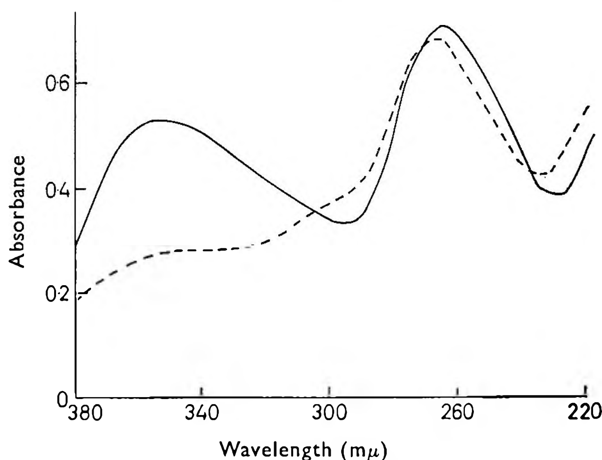
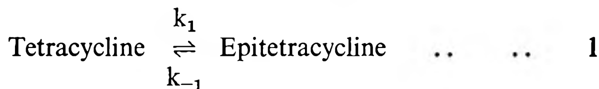


FIG. 1. Ultraviolet spectral curves showing the degradation of a solution of tetracycline hydrochloride at pH 7·0. Assayed in 0·1 N sulphuric acid after zero hr (———) and 1180 hr (- - - -).

increasing buffer strength. Since epimerization proceeds relatively slowly in water, we used acetate buffer systems. With the method described below, for most systems, the rate of epimerization was increased to such an extent that equilibrium mixtures of a tetracycline and its epimer could be obtained before other degradation became significant. This was established by determining the 254/267 and 355/298 ratios. The 254/267 ratio increased to an equilibrium level and remained relatively constant, whereas the 355/298 ratio increased to a point after which it began to decrease steadily, indicating that degradation other than epimerization was becoming significant. Thus, it can serve as a valuable corollary in indicating where the 254/267 ratio could be used validly to follow epimerization. The wavelengths used for the corollary assay varied with the tetracycline analogue examined whereas the 254/267 ratio was used for all the derivatives.

Remmers & others (1963) showed that the epimerization of tetracycline follows the kinetics of a first-order reversible reaction.



$$\text{Tetracycline} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Epitetracycline} \quad .. \quad .. \quad 1$$

The rate coefficients can be obtained using the equation describing the kinetics of such a reaction

$$\ln \frac{A_o - A_e}{A - A_e} = (k_1 + k_{-1})\, t \quad .. \quad .. \quad .. \quad 2$$

where A_o = % tetracycline hydrochloride at time 0 hr; A = % tetracycline hydrochloride at time t hr; A_e = % tetracycline hydrochloride at equilibrium; k = forward rate coefficient, hr^{-1}; k_{-1} = backward rate coefficient, hr^{-1}.

If log $(A - A_e)$ is plotted as a function of time, a straight line should

be obtained whose slope is equal to $-\frac{(k_1 + k_{-1})}{2{\cdot}303}$ (the method of least squares was used to estimate the slope). The individual rate coefficients can then be obtained knowing the equilibrium concentration of tetracycline using the expression:

$$\frac{100 - A_e}{A_e} = \frac{k_1}{k_{-1}} \quad \ldots \quad \ldots \quad \ldots \quad 3$$

TETRACYCLINE AND DEMETHYLCHLORTETRACYCLINE

The values of the forward and backward rate coefficients for the epimerization of tetracycline hydrochloride and demethylchlortetracycline hydrochloride under various experimental conditions are summarized in Tables 1 and 2.

TABLE 1. RATE COEFFICIENTS FOR THE EPIMERIZATION OF TETRACYCLINE HYDROCHLORIDE. (Assays in 0·1N sulphuric acid.)

Acetate buffer strength	pH		Rate coefficients (hr^{-1}) × 10^2		
			30°	37°	50°
0·1M	4·0	k_1	1·8	3·5	13
		k_{-1}	3·7	7·6	39
	5·0	k_1	0·8	1·9	6·9
		k_{-1}	2·3	5·7	33
M	4·0	k_1	14	33	88
		k_{-1}	25	62	170
	5·0	k_1	8·1	14	41
		k_{-1}	20	35	110
	6·0	k_1	1·1	2·7	5·8
		k_{-1}	3·3	9·4	19

TABLE 2. RATE COEFFICIENTS FOR THE EPIMERIZATION OF DEMETHYLCHLORTETRACYCLINE HYDROCHLORIDE. (Assays in 0·1N sulphuric acid.)

Acetate buffer strength	pH		Rate coefficients (hr^{-1}) × 10^2		
			30°	37°	50°
0·1M	4·0	k_1	2·2	4·5	12
		k_{-1}	4·6	10	28
	5·0	k_1	0·9	1·7	4·6
		k_{-1}	2·8	5·8	18
M	4·0	k_1	20	37	110
		k_{-1}	41	76	220
	5·0	k_1	7·9	15	44
		k_{-1}	21	42	120
	6·0	k_1	1·4	2·4	9·4
		k_{-1}	4·2	7·5	29

At pH 4·0 and 5·0 the epimerization progressed rapidly enough to allow an equilibrium to be reached before other degradation became a factor. However, at pH 6·0 in 0·1M buffer, the epimerization proceeded so slowly that it was apparent that other degradation was occurring in most instances before an equilibrium 254/267 ratio was reached.

The epimerization of tetracycline and demethylchlortetracycline proceeded fastest and to the greatest extent at pH 4·0, which was the lowest pH value studied. Remmers (1963) reported that epimerization took place most rapidly at a pH between 3·0 and 4·0. The equilibrium mixtures for both tetracycline and demethylchlortetracycline at pH 4·0 contained

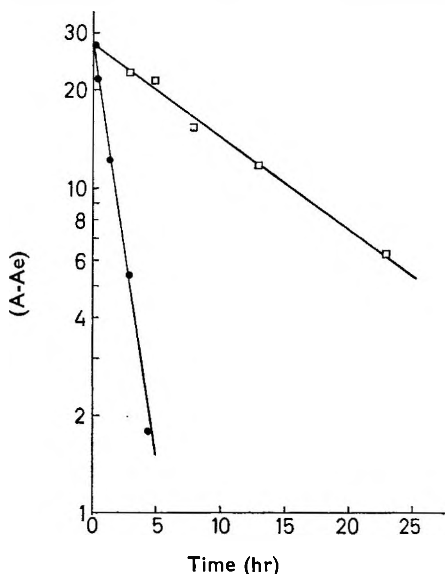


FIG. 2. The influence of buffer concentration on the epimerization of demethylchlortetracycline at pH 4·0 and 30° C. Assays were conducted in 0·1 N sulphuric acid. □ 0·1 M acetate buffer. ● M acetate buffer. See text p. 541 for definition of A–Ae.

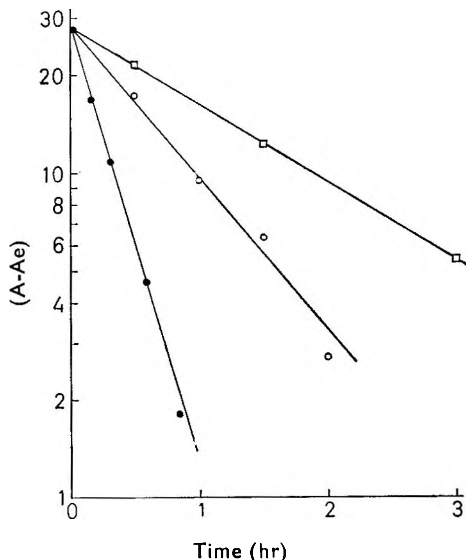


FIG. 3. The influence of temperature on the epimerization of demethylchlortetracycline in a pH 4·0 M acetate buffer. Assays were conducted in 0·1 N sulphuric acid. □ 30°. ○ 37°. ● 50° C.

approximately 34% of the corresponding epimer whereas, at pH 5·0, approximately 25% of the epimer was present at equilibrium. The epimerization proceeded slightly further when the M buffer was used.

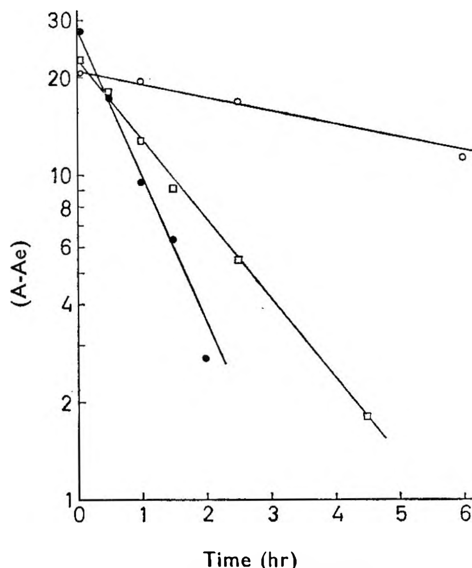


FIG. 4. The influence of pH on the epimerization of demethylchlortetracycline in M acetate buffer at 37°C. Assays were conducted in 0·1 N sulphuric acid. ● pH 4·0. □ pH 5·0. ○ pH 6·0.

Buffer concentration and temperature influenced the rate of epimerization (Tables 1 and 2). The influence of buffer concentration, temperature and pH have been illustrated in Figs 2–4.

TABLE 3. ENERGIES OF ACTIVATION FOR THE EPIMERIZATION OF TETRACYCLINE AND DEMETHYLCHLORTETRACYCLINE

Acetate buffer strength	pH	Reaction	Tetracycline E_a kcal/mole	Demethylchlortetracycline E_a kcal/mole
0·1M	4·0	Forward	19	16
		Backward	23	17
	5·0	Forward	21	16
		Backward	26	18
M	4·0	Forward	18	17
		Backward	18	17
	5·0	Forward	16	17
		Backward	17	17
	6·0	Forward	16	19
		Backward	16	19

The energies of activation for the epimerization of tetracycline and demethylchlortetracycline have been determined using the Arrhenius equation,

$$\log k = \frac{-E_a}{2{\cdot}303\,RT} + \log s \quad .. \quad .. \quad .. \quad 4$$

where k is the rate coefficient, E_a is the energy of activation, R is the gas constant, T is the absolute temperature and s is a constant referred to as the frequency factor. These values have been tabulated in Table 3. The energy of activation obtained for tetracycline when a pH 4·0, 0·1M

acetate buffer is used compares with the values obtained by Remmers & others (1963) using a phosphate buffer (20·4 kcal/mole for both the forward and backward reactions).

CHLORTETRACYCLINE

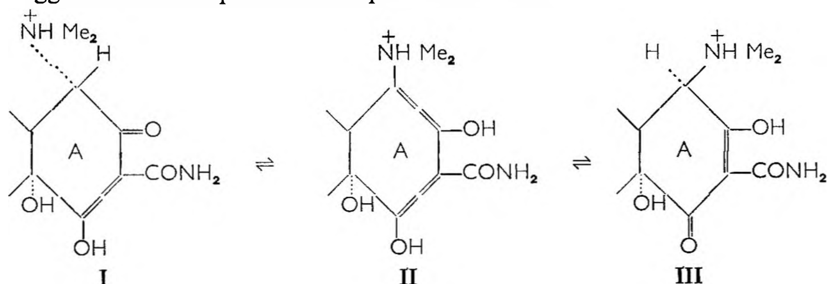
Chlortetracycline is unstable in aqueous solutions; we found that degradation other than epimerization occurs rapidly, even at pH 4·0.

OXYTETRACYCLINE

Epimerization of oxytetracycline occurred very slightly, or not at all. The 254/267 ratio remained relatively constant, whereas the 353/298 ratio eventually decreased, indicating other degradation was taking place.

Jarowski (1963) stated that tetracycline will epimerize much more easily than oxytetracycline. He suggested that the hydroxyl group at position 5 of oxytetracycline could hydrogen bond with the dimethylamino-group and thus inhibit the tendency to epimerize. By this reasoning, tetracycline and demethylchlortetracycline, which lack this hydroxyl group, would show a greater tendency to epimerize. This would satisfactorily explain our findings.

Recently, Huettenrauch & Keiner (1966) investigated the influence of structural modification at C-2 on the epimerization of several tetracycline derivatives. They described the epimerization of *N*-(pyrrolidinomethyl)-tetracycline and *N*-(pyrrolidinomethyl)oxytetracycline and reported that, after cleavage to form tetracycline and oxytetracycline, respectively, the oxytetracycline epimerized more readily. This conflicts with our results although acetic acid was used and, by acting as a stronger protonating solvent than water, it should promote the rearrangement of ring A (on which the dimethylamino-group is a substituent) so as to favour the epimerization process. For example, the following scheme could be suggested for the epimerization process in acid:



Rigler, Bag & others (1965) have suggested the above structures for tetracycline (I) and its epimer (III). These authors also noted striking differences in the basicities of the nitrogen atoms (of the dimethylamino-group) of the two epimers and indicated that the difference in conformation of ring A may be partly responsible for this.

As well as promoting the rearrangement of ring A, the acetic acid may interact with the hydroxyl group at position 5 of the oxytetracycline, thus competing with the bonding of this hydroxyl group with the dimethylammonium group. This would result in at least partial removal of this

inhibition of the epimerization process and therefore, in acetic acid, the 5-hydroxy group may not interfere with the epimerization.

THE INFLUENCE OF CALCIUM ON EPIMERIZATION

Above pH 6·0 epimerization of tetracycline occurs only slightly and at a very slow rate. Kaplan, Granatek & Buckwalter (1957) reported that epimerization is markedly inhibited by calcium or magnesium above a pH of 6.

We found that in the presence of calcium the rate of epimerization was unchanged at pH values below 6·0 even when a 5:1 calcium:tetracycline concentration ratio was used. These results are not surprising in the light of potentiometric studies previously made in these laboratories in which the formation of calcium–tetracycline complexes was not observed below a pH of 6·0. Thus, for calcium to inhibit the epimerization process, it may be necessary for it to complex with tetracycline.

THE INFLUENCE OF COPPER ON EPIMERIZATION

Since calcium had no influence on the rate of epimerization of tetracycline over the pH range examined, the effects of a metal having different complexation characteristics at these pH values was studied. Copper–tetracycline molar ratios ranging from 1:5 to 2:1 were used. It was found that degradation other than epimerization occurred at a faster rate than it did when copper was not present in the system. Thus, it would appear that copper promotes the degradation of the tetracyclines.

Kaplan, Lannon & Buckwalter (1965) reported the inactivation of tetracycline with a cupric–morpholine complex but stated that cupric ion as cupric acetate, or copper tyrosinase did not inactivate tetracycline. Although the biological activity of the solutions used by us was not measured, it was apparent that degradation was occurring rapidly in the presence of copper.

Since it was the purpose of this study to investigate the epimerization process, the other routes of degradation were not analyzed.

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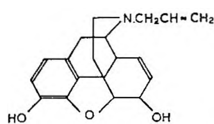
Interactions of narcotic antagonists and antagonist-analgesics

G. F. BLANE AND D. DUGDALL

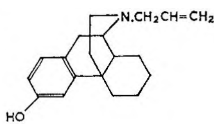
In the rat the potent narcotic antagonist *N*-cyclopropylmethyl-6,14-*endo*ethano-7α-(1-hydroxy-1-methylethyl)-tetrahydronororipavine (M5050, Reckitt), which itself lacks analgesic activity, resembled naloxone in its capacity to reverse the antinociceptive effects of morphine antagonist-analgesics. The nociceptive stimulus employed was bradykinin administered by an intra-arterial route. ED50 values were established for the reversal by M5050 of the analgesic effect of nalorphine, levallorphan, pentazocine and some newer compounds. When antagonist-analgesics were given concomitantly with morphine the response varied from antagonism of the analgesic effect of the morphine to synergism, depending on the dose combination.

THE narcotic antagonist naloxone differs from well-established morphine antagonists, such as nalorphine and levallorphan, in possessing no antinociceptive properties of its own in animal tests (Blumberg, Wolf & Dayton, 1965). Again, unlike nalorphine, naloxone does not produce dose-related analgesic effects in man, nor is its use associated with respiratory depression or psychotomimetic side-effects (Foldes, 1964; Lasagna, 1965; Sadove, Balagot & others (1963). Recently, Blumberg, Dayton & Wolf (1966) observed that naloxone also antagonizes the analgesic effects of nalorphine, pentazocine, cyclazocine, cyclorphan and levallorphan in mice and rats using intraperitoneal phenyl-*p*-benzoquinone as the nociceptive agent.

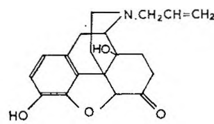
One compound from an extensive series of bridged-ring derivatives of tetrahydrothebaine (Bentley, 1967) showed a preliminary pharmacological profile sufficiently similar to naloxone to stimulate our making a comparison. The lack of antinociceptive activity of *N*-cyclopropylmethyl-6,14-*endo*ethano-7α-(1-hydroxy-1-methylethyl)-tetrahydronororipavine(M5050, Reckitt) in the rat tail-pressure, the mouse phenyl-*p*-benzoquinone and the rat bradykinin tests has already been reported (Blane, 1967).



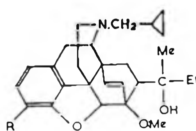
Nalorphine



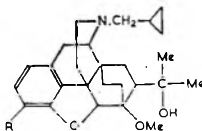
Levallorphan



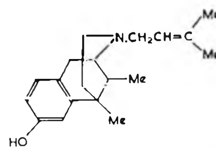
Naloxane



M 5217 R=OH
M 5205 R=OMe



M 5050 R=OH
M 5046 R=OMe



Pentazocine

From the Pharmacology Laboratory, Reckitt and Sons Ltd., Hull, England.

We have now used the bradykinin test in the rat, first to compare M5050 with naloxone, and subsequently to examine the interaction of M5050 with antagonist-analgesics, some of which themselves are new (see formulae).

The interaction of the antagonist-analgesics nalorphine and pentazocine with morphine has also been examined.

Experimental

Antinociception in the rat. Male SPF-derived Sprague–Dawley rats of between 250 and 350 g were used in groups of 5 or 10. The minimum dose of intra-arterial bradykinin required to consistently produce dextro-rotation of the head and flexion of the right fore-limb was established for each rat and subsequently repeated at regular intervals to provide the basis for a time-response curve. Disappearance of this syndrome after drug administration was taken as the criterion for scoring an analgesic effect. Details of this procedure were given by Blane (1967).

Antinociception in the mouse. Female mice (C.D. strain) were used in groups of 5 or 10. Antinociceptive activity was determined as the dose of drug required to reduce by 50% the number of abdominal stretches (writhes) caused by intraperitoneal injection of 2 mg/kg of phenyl-*p*-benzoquinone (Hendershot & Forsaith, 1959).

Narcotic antagonist activity. Antagonist activity was assessed as that dose of drug in the rat tail-pressure test, which reduced to 50% the total analgesia caused by 10 mg/kg morphine sulphate administered subcutaneously (Green, Ruffell & Walton, 1954).

Interaction of antagonists with antagonist-analgesics. The bradykinin test was used. Doses were based on preliminary findings. When antagonists of the naloxone type were evaluated against antagonist-analgesics all animals initially received a dose of the antagonist-analgesic which was nearest to that blocking the bradykinin-induced syndrome in 9 out of 10 animals (ED 86–94). Groups of animals also received at the same time one of a series of doses of the antagonist under test. The results were expressed in terms of the dose of antagonist required to effect a 50% reduction in the number of animals showing analgesia at the time of the peak effect, i.e. from about 86–94% to about 43–47%.

Interaction of morphine with nalorphine and pentazocine. The response to bradykinin of rats given an analgesic ED20 of morphine followed immediately by one of a graded series of doses of nalorphine was assessed. So too was the response of animals given an analgesic ED20 of nalorphine and different doses of morphine. Similar experiments were made using morphine and pentazocine.

Statistical examination of data. Agonist ED50 values and their 95% confidence limits were estimated using the method of Litchfield & Wilcoxon (1949). ED50 values in the interaction studies were estimated (Litchfield & Wilcoxon) from the regression lines relating reduction in the number of animals showing analgesia to the logarithm of the dose of antagonist. ED50 values were always assessed at the time of peak drug or drug mixture effect.

Drugs. Morphine, levallorphan, pentazocine and the Reckitt compounds: M5046 [*N*-cyclopropylmethyl-6,14-*endo*ethano-7α-(1-hydroxy-1-methylethyl)-tetrahydronorthebaine], M5050 [*N*-cyclopropylmethyl-6,14-*endo*ethano-7α-(1-hydroxy-1-methylethyl)-tetrahydronororipavine], M5205 [*N*-cyclopropylmethyl-6,14-*endo*etheno-7α(1-(*R*)-hydroxy-1-methylpropyl)-tetrahydronorthebaine] and M5217[*N*-cyclopropylmethyl-6,14-*endo*theno-7α-(1-(*R*)-hydroxy-1-methylpropyl)-tetrahydronororipavine], the chemistry of which have been described by Bentley (1967).

All drugs, other than bradykinin, were administered subcutaneously. Where the interaction of two drugs was studied in a single animal, both drugs were injected simultaneously into different sites. All doses are expressed as the weight of the salt. Morphine was used as the sulphate, nalorphine as the hydrobromide, levallorphan as the tartrate. The other compounds were hydrochlorides. Bradykinin was given as a solution of the pure synthetic peptide (Sandoz) in 0·9% saline.

Results

Reversal by naloxone and M5050 of the antinociceptive effects of antagonist-analgesics. Naloxone, like M5050, had no activity at doses up to 100 mg/kg in antinociception tests. Each drug was then administered in

TABLE 1. ANTAGONISM OF NALORPHINE ANALGESIA BY NALOXONE AND M5050 IN THE BRADYKININ TEST. The dose of nalorphine is, to the nearest mg, that which when given alone blocks the response to bradykinin in 9 out of 10 animals (ED87). All drugs given subcutaneously.

	Naloxone			M5050		
Nalorphine mg/kg	Drug mg/kg	Block of nociception %	ED50 mg/kg (95% limits)	Drug mg/kg	Block of nociception %	ED50 mg/kg (95% limits)
10	1·0	70 (7/10)	4·1 (2·1–7·6)	0·005	80 (8/10)	0·016 (0·010–0·024)
10	2·5	60 (6/10)		0·010	60 (6/10)	
10	5·0	40 (4/10)		0·020	40 (4/10)	
10	10·0	20 (2/10)		0·030	20 (2/10)	

TABLE 2. INTERACTION OF M5050 WITH ANTAGONIST-ANALGESICS. Drugs are ranked in descending order of morphine antagonist activity.

Antagonist-analgesic	Analgesic activity, s.c. ED50 (mg/kg)		Morphine antagonist activity in rats ED50 (mg/kg)	Reversal of anti-bradykinin action in rats by M5050 ED50 (mg/kg) 95% confidence limits in parentheses
	Anti-writhing (mice)	Anti-bradykinin (rats)		
M5217	0·029	0·39	0·021	0·135 (0·090–0·198)
Levallorphan	2·4	125·0	0·30	0·120 (0·053–0·270)
Nalorphine	2·1	4·0	0·48	0·016 (0·010–0·026)
M5046	1·5	2·4	5·4	0·008 (0·003–0·015)
Pentazocine	3·0	1·85	≃30	0·013 (0·009–0·018)
M5205	2·5	2·5	44	0·008 (0·004–0·018)

a graded series of doses to rats at the same time as sufficient nalorphine to abolish the response to intra-arterial bradykinin in 9 out of 10 animals. Nalorphine analgesia was antagonized by M5050 and naloxone in a dose-related manner (Table 1); M5050 was more than 250 times the more potent. M5050, in a graded series of doses, was then evaluated in rats given doses close to the ED90 of antagonist-analgesic compounds including levallorphan and pentazocine, as well as M5046, M5205 and M5217.

Table 2 shows the antinociceptive and morphine-antagonist actions of these compounds, as well as the ED50 values for reversal of analgesia by M5050 in the bradykinin test. Values for antagonism of analgesia caused by pentazocine, M5046 and M5205 were similar to that already established against nalorphine analgesia, and ranged between 0·008 and 0·013 mg/kg. However, we found levallorphan and M5217 analgesia to be less readily antagonized by M5050, the ED50 values for reversal of analgesia being 0·120 mg/kg and 0·135 mg/kg respectively. Blumberg & others (1966) also reported a high ED50 for reversal of levallorphan analgesia, using naloxone.

TABLE 3. EFFECT OF MIXTURES OF MORPHINE AND NALORPHINE ON BRADYKININ-INDUCED NOCICEPTION IN THE RAT. Drugs given synchronously subcutaneously. Analgesic values represent means for block of nociception in groups of at least 10 animals.

Experimental design	Morphine (A) Dose (mg/kg)	Morphine (A) Expected analgesia %	Nalorphine (B) Dose (mg/kg)	Nalorphine (B) Expected analgesia %	A + B Observed/ expected analgesia* %	Comment
Nalorphine dose fixed, morphine dose varied	0·1	<1	3·0	20	30/<21	Synergism
	0·2	3	3·0	20	60/23	Synergism
	0·4	10	3·0	20	80/30	Synergism
	0·6	20	3·0	20	80/40	Synergism
Morphine dose fixed, nalorphine dose varied	0·6	20	0·1	0	0/20	Antagonism
	0·6	20	0·5	<1	20/<21	Addition
	0·6	20	1·0	<1	30/20	Synergism ?
	0·6	20	2·0	7	40/27	Synergism
	0·6	20	3·0	20	80/40	Synergism

* Analgesia expected on the basis of a simple additive effect of the two drugs.

Interaction of antagonist-analgesics with morphine. Mixtures of morphine and nalorphine were given to rats receiving analgesic doses of bradykinin at regular intervals. Table 3 shows that a range of doses of morphine, which would be expected to cause from less than 1% up to 20% analgesia in this test, acted synergistically with a dose of nalorphine (3 mg/kg) which would itself produce analgesia in 20% of animals. When the morphine dose was fixed at the 20% analgesia level (0·6 mg/kg) and the nalorphine dose varied from the zero to 20% effect, the overall effect changed from apparent antagonism with the lowest dose of nalorphine, through simple addition, to synergism with the 3 mg/kg nalorphine dose. However, except at one or two points, the synergism was not highly significantly different from an expected value for simple additive effect ($P \simeq 0{\cdot}05$) in either experiment. Even very large doses of nalorphine did not cause 100% of animals to show analgesia.

Like nalorphine, pentazocine in very low doses (0·35 mg/kg) antagonized the analgesic effect of an ED20 or morphine. Intermediate dose levels

of pentazocine (0·63 and 1·25 mg/kg) acted additively with morphine, but with the 2·5 and 5·0 mg/kg doses of pentazocine there was a return to antagonism (Table 4).

TABLE 4. EFFECT OF MIXTURES OF MORPHINE AND PENTAZOCINE ON BRADYKININ-INDUCED NOCICEPTION IN THE RAT. Drugs given synchronously subcutaneously. Analgesic values represent means for block or nociception in groups of at least 10 animals.

Experimental design	Morphine (A)		Pentazocine (B)		A + B Observed/ Expected analgesia* %	Comment
	Dose (mg/kg)	Expected analgesia %	Dose (mg/kg)	Expected analgesia %		
Morphine dose fixed, pentazocine dose varied	0·6	20	0·35	10	0/30	Antagonism
	0·6	20	0·63	20	40/40	Addition
	0·6	20	1·25	40	60/60	Addition
	0·6	20	2·5	80	40/100	Antagonism
	0·6	20	5·0	60	0/80	Antagonism

* Analgesia expected on the basis of a simple additive effect of the two drugs.

Discussion

Our findings with naloxone and also with M5050 confirm the report of Blumberg & others (1966) that morphine antagonists of this type, which themselves lack analgesic activity, are capable of reversing the antinociceptive effects of antagonist-analgesics, as well as an agonist-analgesic such as morphine. M5050 was exceptionally potent in this respect.

TABLE 5. SUMMARY OF ACTIONS OF ANTAGONIST-ANALGESICS

Antagonist-analgesic	Reversal of anti-bradykinin action in rats by M5050 ED50 (mg/kg)	Ratio: Analgesia in bradykinin-rat / Morphine antagonism in rat tail
Levallorphan	0·120	416·0
M5217	0·135	18·6
Nalorphine	0·016	8·3
M5046	0·008	0·44
Pentazocine	0·013	0·062
M5205	0·008	0·057

The values for reversal by M5050 of the analgesia caused by antagonist-analgesics in the bradykinin test may be used as a basis to divide these compounds into two categories (Table 5); firstly, compounds having an analgesic action which is reversed by a low dose of M5050 (0·008–0·016 mg/kg), including nalorphine and pentazocine which are known to have an analgesic action in man; secondly, compounds for which the reversal ED50 is approximately 10 times greater, including levallorphan, which appears to lack useful analgesic activity in man (Foldes, 1964; Keats & Telford, 1966, 1967). The ED50 values within these groups are statistically similar but there is a high level of significance ($P < 0·001$) to the difference between any member of one group and any member of the other group. The figures in the second column of Table 5 represent the ratio analgesia (as assessed in the bradykinin test): morphine antagonism; the compounds are ranked in descending order of this ratio.

The compounds for which this ratio is high—levallorphan and M5217—are those which had high values for reversal of analgesia by M5050. By contrast, the analgesia: antagonism ratio is low for the remaining antagonist-analgesics which were reversed by a low dose of M5050.

We interpret this to suggest that levallorphan and M5217 have high receptor affinity with low analgesic efficacy. As such they are intermediate between the other antagonist-analgesics and the antagonists, such as M5050, which have very high receptor affinity with negligible analgesic efficacy.

Low doses of nalorphine and pentazocine antagonized the analgesic effect of morphine in the bradykinin test while with higher doses there was evidence of summation and perhaps synergism between the analgesic actions of the two types of drugs. Houde, Wallenstein & others (1967) report that appropriate morphine-pentazocine combinations caused more analgesia in cancer patients than either drug alone. At least with pentazocine, in our rats still higher doses resulted in fewer, and eventually no animals showing analgesia, which suggests that this drug can antagonize itself as the dose is increased beyond a certain threshold.

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Influence of 1-(5-methyl-1-phenylpyrazol-4-yl)-3-[4-(*o*-tolyl)piperazin-1-yl]-propan-1-one hydrochloride (CIBA 1002-Go) on the stores of catecholamine in rat and cat tissues

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The effect of 1002-Go has been examined on the catecholamine stores in rat heart, brain, adrenals and cat heart. There is a significant reduction in the catecholamine content from all the tissues with doses ranging from 2·5–30 mg/kg. Guanethidine is slightly less potent as a depletor of catecholamine than 1002-Go in the rat. In cats, however, 1002-Go is much weaker than guanethidine or reserpine in depleting the catecholamine stores. There also seem to be differences among these anti-hypertensive agents in the duration of depletion and repletion of the catecholamine stores.

ANTIHYPERTENSIVE drugs differ in their mechanisms of action. Reserpine produces a profound and persistent alteration of the cellular mechanism for binding catecholamines (Green, 1962). α-Methyl-dopa depletes noradrenaline by a different mechanism; the drug is metabolized to α-methylnoradrenaline and this displaces noradrenaline from the binding sites (Andén, 1964). Guanethidine and bretylium block the postganglionic adrenergic transmission and possess a slight and transient ganglion-blocking activity. It has been suggested that they prevent the liberation of adrenergic transmitter from the nerve endings (Boura & Green, 1959; Maxwell, Mull & Plummer, 1959; Maxwell, Plummer & others, 1960). Guanethidine depletes catechol-amine and this has been related to sympathetic blockade (Shepherd & Zimmerman, 1959; Cass, Kuntzman & Brodie, 1960). There is, however, evidence that the onset of sympathetic blockade does not parallel the rate of depletion of catecholamines (Cass & Spriggs, 1961; Sanan & Vogt, 1962).

The drug 1002-Go is a synthetic antihypertensive agent belonging to a group of phenyl piperazine Mannich products (Arya, Grewal & others, 1967). It lowers the blood pressure of renal hypertensive rats to normo-tensive level when given at 5–10 mg/kg twice daily (Grewal, Kaul & David, 1968). 1002-Go produces reversal of the effects of adrenaline at 0·25–0·5 mg/kg without any significant change in the noradrenaline pressor response. The compound blocks amphetamine and tyramine pressor responses in anaesthetized cats and dogs, an observation which suggests an interference with the release of catecholamines from the nerve endings (Burn & Rand, 1958). This block of amphetamine and tyramine pressor response is not related to the adrenolytic activity of the compound as 1002-Go has a very weak α-adrenergic blocking activity judged by diminution of noradrenaline response on blood pressure and aortic strips (Grewal & Kaul, unpublished observations). Since 1002-Go inhibits the pressor responses of amphetamine and tyramine, and many antihypertensive drugs are known to interfere with the release or normal distribution of the neurotransmitter at the sympathetic myoneural

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junction, it was interesting to see the effect of 1002-Go on the catecholamine stores in rat and cat tissues.

Experimental

METHODS

Male rats, from 140 to 160 g were used. Extraction of the tissues was by perchloric acid. The catecholamines were adsorbed on acid-washed alumina, pH 8·4 and eluted with 0·2N acetic acid as described by Crout, Creveling & Udenfriend (1961). For the assay of adrenals, the total extract was used. Noradrenaline from heart and brain was assayed on the blood pressure of a pithed rat using noradrenaline as a standard. The total catecholamines from adrenals were assayed on the spinal cat (blood pressure) using adrenaline as a standard.

Effect of 1002-*Go on the uptake of noradrenaline by heart.* The method used was that of Muscholl (1961) except that the rats were not pithed. Male rats from 140–160 g were treated with 5 mg/kg (i.p.) of 1002-Go. After 3 hr the rats were anaesthetized with urethane (15% 1·5 ml/100 g body weight). The jugular vein was cannulated and an infusion of noradrenaline (20 μg) was given at a constant rate for 20 min, using a motor-driven syringe. The total volume injected in any one experiment was not more than 2·5 ml. The rats were killed 5 min after the end of the infusion and noradrenaline from the heart was estimated on the blood pressure of the pithed rat. Control experiments were also made in which normal rats anaesthetized with urethane were given 20 μg of noradrenaline by infusion. These rats were killed 5 min after the end of the infusion and noradrenaline content of the heart was estimated.

In these uptake experiments infusion of noradrenaline was made in such a way that each rat was never anaesthetized for more than 1 hr and during this period urethane anaesthesia produces little change in the catecholamine content of the heart (Spriggs, 1965). The recoveries of added noradrenaline to tissue were 60–70%. Values reported are not corrected for the recoveries.

A solution of 1002-Go was prepared in warm polyethylene glycol. An equivalent volume of polyethylene glycol was given to control rats. Each series of experiments had its own controls. To minimize the likelihood of various factors influencing the results, a random order of treatment with the drug and the control solutions and subsequent procedures was used. All injections were made intraperitoneally.

Drugs used. (—)-Noradrenaline hydrogen (+)-tartrate, (—)-adrenaline hydrogen (+)-tartrate. Stock solutions of these two drugs were made in normal saline with 0·1N hydrochloric acid and further dilutions were made from the stock solutions. All concentrations and doses of noradrenaline refer to its salt, but the concentration of adrenaline refers to free base. Guanethidine was used as its sulphate.

Results

The effect of 1002-Go and guanethidine on the rat heart catecholamine content is shown in Table 1. It can be seen that a significant fall in rat

TABLE 1. *In Vivo* EFFECT OF 1002-GO AND GUANETHIDINE ON THE CATECHOLAMINE CONTENT OF THE RAT HEART AT DIFFERENT TIME INTERVALS AFTER TREATMENT

Treatment	Dose mg/kg	Time after treatment (hr)	Catecholamine conc. (μg/g ± s.e.†)
Controls ..	—	—	0·73 ± 0·03 (35)
1002-Go ..	2·5	1	0·59 ± 0·05 (9)*
		3	0·43 ± 0·05 (11)**
		6	0·54 ± 0·06 (7)**
		12	0·72 ± 0·05 (8)
Controls ..	—	—	0·67 ± 0·03 (35)
1002-Go ..	5	1	0·55 ± 0·03 (10)**
		3	0·18 ± 0·05 (7)***
		6	0·13 ± 0·02 (7)***
		12	0·67 ± 0·08 (8)
Controls ..	—	—	0·71 ± 0·04 (24)
Guanethidine..	5	1	0·68 ± 0·058 (4)
		3	0·26 ± 0·06 (6)***
		6	0·30 ± 0·04 (8)***
		12	0·41 ± 0·05 (5)***

Figures in parentheses show the number of animals used.
† Values are not corrected for recoveries.
* 0·05 > P > 0·01. ** 0·01 > P > 0·001. *** 0·001 > P.

heart catecholamine content occurred within 1 hr of treatment with 2·5 and 5 mg/kg of 1002-Go. At 3 hr both doses showed a highly significant effect, the onset of which was quite rapid with a return to normal levels within 12 hr. Guanethidine (5 mg/kg) seems to be slightly less potent as a catecholamine depletor than 1002-Go on the rat heart (Table 1).

A significant depletion of brain noradrenaline was effected by 10 mg/kg of 1002-Go 3 hr after the drug. The concentrations in μg/g were: control 0·40 ± 0·03; drug 0·10 ± 0·002 (0·001 > P). A dose of 30 mg/kg was necessary to produce a significant depletion of the adrenals. After 3 hr, the concentrations in μg/g were: control 969·9 ± 105; drug 514·4 ± 47 (0·01 > P > 0·001).

TABLE 2. *In Vivo* EFFECT OF 1002-GO, GUANETHIDINE AND RESERPINE ON THE CATECHOLAMINE CONTENT IN THE CAT HEART AT DIFFERENT TIME INTERVALS AFTER TREATMENT

Treatment	Dose mg/kg	Time after treatment (hr)	Catecholamine conc. (μg/g ± s.e.†)
Control ..	—	—	1·49 ± 0·11 (22)
1002-Go ..	5	3	1·38 ± 0·26 (4)
		6	1·60 ± 0·19 (8)
		12	1·26 ± 0·14 (8)
1002-Go ..	10	3	1·07 ± 0·05 (3)
		6	1·07 ± 0·11** (4)
		12	1·08 ± 0·11** (3)
1002-Go ..	20	3	0·85 ± 0·12*** (5)
		6	0·65 ± 0·04*** (3)
		12	0·80 ± 0·06*** (8)
Guanethidine	5	3	0·64 ± 0·15*** (3)
		6	0·57 ± 0·09*** (4)
		12	0·30 ± 0·07*** (6)
Reserpine ..	1	16	0·04 ± 0·008*** (4)

Figures in parentheses show the number of animals used.
† Values are not corrected for recoveries.
** 0·01 > P > 0·001. *** 0·001 > P.

The effect of 1002-Go, guanethidine and reserpine on the cat heart is shown in Table 2. There was a significant reduction in the catecholamine content at 3, 6 and 12 hr after 20 mg/kg of 1002-Go, and at 6 and 12 hr with the 10 mg/kg dose.

Reserpine (1 mg/kg) 16 hr after treatment caused a 97% depletion of catecholamine from the cat heart. Guanethidine (5 mg/kg) produced 61 to 81% reduction in the catecholamine content (Table 2). Thus guanethidine and reserpine would seem to be more potent than 1002-Go as catecholamine depletors on this preparation.

Table 3 shows the effect of 1002-Go on the uptake of infused noradrenaline by the rat heart. The means for the noradrenaline content in heart after the infusion are slightly lower in 1002-Go-treated rats than the controls, which suggests that 1002-Go interferes with the uptake of infused noradrenaline.

The effect of 1002-Go after long term treatment on the rat heart is shown in Table 4. Given for 10 days or given only once, 1002-Go (1 mg/kg) produced a similar reduction in the noradrenaline levels in the rat heart.

TABLE 3. EFFECT OF 1002-GO ON THE NORADRENALINE UPTAKE BY THE HEART AFTER AN INFUSION OF NORADRENALINE

Treatment	µg of noradrenaline infused in 20 min	Concentration of noradrenaline in µg/g
Control	—	0·59 ± 0·04 A
1002-Go, 5 mg/kg ..	—	0·18 ± 0·05 B
Control	20 µg	1·08 ± 0·11 C
1002-Go, 5 mg/kg ..	20 µg	0·48 ± 0·03 D

A highly significant difference was found between A and B, A and C, C and D but no significant difference between A and D.

TABLE 4. *In Vivo* EFFECT OF 1002-GO ON THE CATECHOLAMINE CONTENT OF THE RAT HEART

Treatment	Dose and time	Catecholamine conc. (µg/g ± s.e.*)
Controls		0·88 ± 0·03 (5)
1002-Go	1 mg/day for 10 days	0·58 ± 0·04*** (5)
Controls		0·75 ± 0·08 (5)
1002-Go	1 mg	0·47 ± 0·08 (5)†

Figures in parentheses show the number of animals used.

* Values are not corrected for recoveries.
† 0·05 > P > 0·01.
*** 0·001 > P.

Discussion

The results show that 1002-Go, like many other antihypertensive drugs, depletes catecholamine in the rat and cat tissues. The depletion it

causes differs from reserpine and guanethidine in that its onset of action and the recovery of catecholamine is much faster than seen with reserpine and guanethidine (Cass & others, 1960; Orlans, Finger & Brodie, 1960). One hr after treatment, 1002-Go produced a significant fall in the catecholamine content of rat heart at 2·5 and 5 mg/kg. At 6 hr the effect was maximum and the normal levels were reached after 12 hr (Table 1). With guanethidine a highly significant effect was not observed until after 3 hr and the values were significantly lower than the controls even up to 12 hr, although the values tended to return to pretreatment levels after that time. These results are in agreement with those of Bogaert, De Schaepdryver & De Vleeschhouwer (1961) who found 53% depletion of catecholamine in the rat hearts 6 hr after 8 mg/kg of guanethidine and no effect after 24 hr. Cass & Spriggs (1961) have also shown 80–90% depletion of heart noradrenaline after guanethidine and normal levels were reached by 48 hr. The earlier recovery of catecholamine after guanethidine observed by Bogaert & others (1961) and by us may be because Cass & Spriggs (1961) used a higher dose of guanethidine (15 mg/kg) and secondly guanethidine was administered subcutaneously which might account for the longer duration of action.

The significant fall in brain noradrenaline at 10 mg/kg of 1002-Go and the significant lowering in the catecholamine content of the adrenals at 30 mg/kg are effects similar to, but less potent than, those of reserpine (Kirpekar, Goodlad & Lewis, 1958; Orlans & others, 1960). The failure of guanethidine to produce any significant change in the brain is not surprising because due to its low lipid solubility it is unlikely to cross the blood brain barrier readily (Cass & others, 1960).

On the cat heart a significant fall in the catecholamine content was observed only with the two higher doses of 1002-Go (10 and 20 mg/kg), but guanethidine and reserpine produced a marked fall in the catecholamine content even at low doses (5 and 1 mg/kg respectively).

Under our experimental conditions guanethidine seems to be less potent as a catecholamine depletor than 1002-Go in rats (Table 1), but both reserpine and guanethidine are more powerful than 1002-Go on the cat heart (Table 2). There also seems to be some difference among these three antihypertensive agents in the duration of depletion and repletion of catecholamine stores. 1002-Go has the shorter duration of action.

The rate of uptake of infused noradrenaline from the circulating blood is less in the presence of 1002-Go than in the controls; however, the block was not complete as is the case for reserpine (Muscholl, 1961).

The decrease in the catecholamine content seen after 1002-Go could be either due to the release of the amine or to the blocking of the synthesis. The depletion of catecholamines observed after long term treatment with 1002-Go, 1 mg/kg, is roughly the same as seen after a single dose (Table 4). This is probably because its effect does not last for a long time and repletion of catecholamine stores occurs rapidly. Our results would therefore suggest that depletion of catecholamine does not play a major role in the hypotensive effect of the compound.

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Hypoglycaemic activity of 1,1′-biadamantylaryl sulphonylureas

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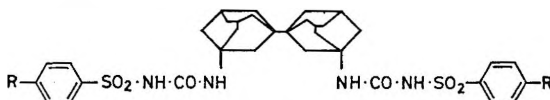
Replacement of the 1-adamantyl group with a 1,1′-biadamantyl group in the *N*-adamant-1-yl-*N*′-arylsulphonylureas leads to compounds having the same or a doubled hypoglycaemic activity. The new biadamantyl derivatives exert a delayed onset of action compared with that of the corresponding adamantane derivatives. The most promising, 3,3′-di(*N*′-*p*-toluenesulphonylureido)-1,1′-biadamantyl (Compd No. 1) and 3,3′-di(*N*′-*p*-methoxybenzenesulphonylureido)-1,1′-biadamantyl (Compd No. 3) are only slightly toxic and appear not to have other pharmacological activity.

THE interesting hypoglycaemic properties of a number of *N*-adamant-1-yl-*N*′-arylsulphonylureas (I) have recently been reported by Gerzon,

I

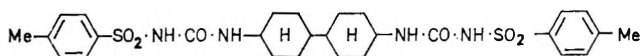
Krumkalns & others (1963). The pharmacological comparison with the corresponding known *N*-alkyl- and *N*-cycloalkyl-*N*′-arylsulphonylureas showed that replacement of either the alkyl or the cycloalkyl group with a 1-adamantyl group yields in certain cases compounds having an equal or greater and more sustained hypoglycaemic action. In particular, *N*-adamant-1-yl-*N*′-*p*-toluenesulphonylurea (Compd No. 2, I; R = Me) has been found to be approximately 15 times more potent than tolbutamide (*N*-butyl-*N*′-*p*-toluenesulphonylurea) and at the same time to have a greatly superior duration of action. All the above compounds are characterized by a rapid onset of action which causes a sudden fall in the blood-sugar level.

Since it is known that polymerization of drugs may lead to an increased or prolonged activity compared to that of the unpolymerized substances, we decided to synthesize three arylsulphonylureas derived from 1,1′-biadamantyl (II) in order to investigate their hypoglycaemic activity and



II

to compare it with that of the corresponding 1-adamantyl derivatives. For the same reasons, but especially for correlation, we have also synthesized an arylsulphonylurea derived from bi(cyclohexyl) (III) comparing



III

its hypoglycaemic activity with that of the corresponding cyclohexyl derivative (glycyclamide; Tolcyclamide).

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Further studies on the more active compounds included toxicity, central nervous system activity and determination of the anticonvulsant, parasympathicolytic, diuretic, antipyretic, choleretic, anticoagulant, anti-infarction, antibacterial, antifungal, and antiparasitic activity.

Chemistry

The compounds in Table 1 were prepared by condensation of *N*-sulphonylcarbamates with amines in boiling toluene. 3,3′-Diamino-1,1′-biadamantyl was obtained from 3,3′-dibromo-1,1′-biadamantyl according to the method, slightly modified, used by Stetter, Mayer & others (1960) to prepare 1-aminoadamantane from 1-bromoadamantane. 4,4′-Diaminobi(cyclohexyl) was prepared by reduction of bi(cyclohexyl)-4,4′-dione dioxime (Wild, Shunk & Hoffman, 1954).

EXPERIMENTAL

3,3′-*Diacetamido*-1,1′-*biadamantyl.* Concentrated sulphuric acid (390 ml) was slowly added to a stirred suspension of 3,3′-dibromo-1,1′-biadamantyl (254 g; Reinhardt, 1962) in acetonitrile (1·9 litres). The suspension was gradually heated to boiling and refluxed for 5 hr, then cooled and poured into ice/water (10 litres). The solid was filtered off, washed repeatedly with water, then with 8% sodium bicarbonate solution, and again with water. After drying, the product was washed with boiling ethanol to give 3,3′-diacetamido-1,1′-biadamantyl (220 g), m.p. 329–330°. Found: C, 74·6; H, 9·3; N, 7·3; $C_{24}H_{36}N_2O_2$ requires C, 74·95; H, 9·4; N, 7·3%.

3,3′-*Diamino*-1,1′-*biadamantyl.* A mixture of 3,3′-diacetamido-1,1′-biadamantyl (164 g) and sodium hydroxide (164 g) in ethylene glycol (2·4 litres) was refluxed with stirring for 5 hr. The resulting solution was poured into water (11 litres), and the precipitate was filtered off and dissolved in chloroform. After drying (Na_2SO_4), the chloroform solution was evaporated and the residue was crystallized from ethanol to give 3,3′-diamino-1,1′-biadamantyl (76 g), m.p. 187–191°. Found: C, 79·9; H, 10·7; N, 9·4; $C_{20}H_{32}N_2$ requires C, 79·9; H, 10·7; N, 9·3%.

Bi(cyclohexyl)-4,4′-*dione dioxime.* A boiling solution of bi(cyclohexyl)-4,4′-dione (24 g) and hydroxylamine hydrochloride (17 g) in ethanol (250 ml) was treated with pyridine (20 g). The resulting suspension was heated under reflux for 30 min, then cooled and filtered. Bi(cyclohexyl)-4,4′-dione dioxime (27 g), m.p. 287–288°, was obtained. Found: C, 64·25; H, 8·9; N, 12·4; $C_{12}H_{20}N_2O_2$ requires C, 64·25; H, 9·0; N, 12·5%.

4,4′-*Diaminobi*(*cyclohexyl*). Platinum oxide (3 g) was added to a solution of the above dioxime (10 g) in glacial acetic acid (350 ml), and the suspension shaken for 15 hr with hydrogen at 20 atmos. pressure. The catalyst was then filtered off, and the solution evaporated to dryness. The residue was dissolved in water, and the solution made alkaline and extracted with ether. The ethereal layer was washed, dried (Na_2SO_4) and evaporated to give 4,4′-diaminobi(cyclohexyl) (5·4 g), m.p. 77–80° unsharp. Found: C, 73·15; H, 12·6; N, 14·0; $C_{12}H_{24}N_2$ requires C, 73·4; H, 12·3; N, 14·3%.

Preparation of the sulphonylureas. The appropriate sulphonylcarbamate (0·12 mole) and amine (0·05 mole) were refluxed in toluene (250 ml) for 5 hr. The hot suspension was filtered and the solid product washed repeatedly with ether and then dried at 130° for 20 hr.

Pharmacology

In all the experiments the blood-sugar level was determined according to Ceriotti (1963).

EFFECT ON THE BLOOD-SUGAR LEVELS IN RATS AND RABBITS

Male Sprague-Dawley rats, 140–160 g, and rabbits, 2·5 kg, all fasted for 18 hr, were used. The drugs were administered orally, suspended in a 5% acacia mucilage. The blood-glucose was determined each hour for 7 hr after administration. The tests were made with 3 doses between 5 and 100 mg/kg and each dose was given to 21 rats and 2 rabbits. The relative hypoglycaemic potency of the drugs was calculated according to Root, Sigal & Anderson (1959). The given relative potency value has been expressed in relation to the hypoglycaemic activity of *N*-adamant-1-yl-*N′*-*p*-toluenesulphonylurea (Compd No. 2), which has been assigned the potency of 1·0.

An analogous experiment was made also on adrenalectomized rats used 18 hr after the operation. Each drug was tested on 21 rats at the single dose of 25 mg/kg, orally. The blood-sugar was determined every 2 hr for 14 hr after administration.

ACTION ON LIVER GLYCOGEN LEVEL IN RATS

Male Sprague-Dawley rats, 200–230 g, fed normally up to the start of the experiment, were used. The animals received 0·71 mmole/kg of compound No. 1 and 3; the liver glycogen (Clementi, 1960) was determined 6 hr later (5 animals/group).

Results

Table 1 shows that compounds Nos. 1 and 3 are approximately just as active, at equal weight doses, as the corresponding adamantane derivatives (compounds Nos 2 and 4), whereas the activity of compound No. 5 is approximately half that of the corresponding adamantane derivative (compound No. 6), all at equal weight. The duration of action was roughly equal for the above compounds. Compound No. 7 was found to be practically inactive.

Fig. 1 shows that when administered to adrenalectomized rats, the new biadamantyl derivatives displayed a hypoglycaemic activity and a total duration of action comparable to that of the corresponding adamantane compounds. The onset of action, however, was different; compounds Nos 1 and 3 brought about the greatest lowering of the blood-sugar level at the 6th hr, whereas compounds Nos 2 and 4 exerted their highest activity at the 2nd hr.

Compounds Nos 1 and 3 significantly increased the liver glycogen in the

TABLE 1. ARYLSULPHONYLUREAS: CHEMICAL CHARACTERISTICS AND HYPOGLYCAEMIC POTENCIES

Compd No.	R	Yield (%)	M.p. °C	Formula	Analyses Found % C	H	N	S	Required % C	H	N	S	Relative potency Rat	Rabbit
1[a] II	*p*-Me	91[b]	325–327	$C_{36}H_{46}N_4O_6S_2$	62·3	6·8	8·1	9·3	62·2	6·7	8·1	9·2	1·05	0·92
2[c] I	*p*-Me	54	173–174	$C_{18}H_{24}N_2O_3S$	62·0	6·9	8·0	9·2	62·0	6·8	8·2	9·4	1·00	1·00
3[a] II	*p*-MeO	89[b]	323–327	$C_{36}H_{46}N_4O_8S_2$	59·4	6·4	7·8	8·7	59·5	6·4	7·7	8·8	0·98	0·95
4 I	*p*-MeO	60[d]	158–159	$C_{18}H_{24}N_2O_4S$	59·7	6·8	7·6	8·6	59·3	6·6	7·7	8·8	0·98	0·96
5[a] II	*p*-Cl	90[b]	336–338	$C_{34}H_{40}Cl_2N_4O_6S_2$	55·7	5·5	7·7	8·7	55·5	5·5	7·6	8·7	0·17	0·15
6[c] I	*p*-Cl	59	149–151	$C_{17}H_{21}ClN_2O_3S$	55·4	5·7	7·6	8·7	55·3	5·6	7·6	8·7	0·35	0·32
7 III	*p*-Me	85[b]	214–217	$C_{28}H_{36}N_4O_6S_2$	56·7	6·2	9·5	10·7	56·9	6·5	9·5	10·9	0·06	0·04
Glycyclamide													0·98	1·03

[a] De Angeli S.p.A. (1966). British Patent Application, 66/48.122.
[b] Crude product.
[c] See Gerzon & others (1963).
[d] Purified according to Gerzon & others (1963).

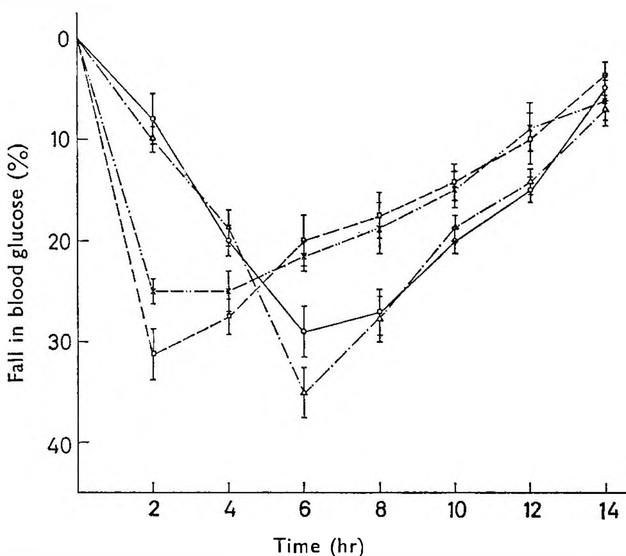

FIG. 1. Hypoglycaemic activity at a dose of 25 mg/kg orally in adrenalectomized rats 3,3′-di(*N*′-*p*-toluene sulphonylureido)-1,1′-biadamantyl (Compd No. 1, ○——○), 3,3′-di(*N*′-*p*-methoxybenzenesulphonylureido)-1,1′-biadamantyl (Compd No. 3, △ — · — · △), *N*-adamant-1-yl *N*′-*p*-toluenesulphonylurea (Compd No. 2, □ — — — □), and *N*-adamant-1-yl-*N*′-*p*-methoxybenzenesulphonylurea (Compd No. 4, ×– – · · – – · · ×). The individual points show the mean glucose levels, while the bars represent the stand errors.

rat (mucilage 35·28 ±0·25 liver glycogen mg/g ±s.e. wet tissue; compd 1, 53·60 ±0·93, P <0·001; compd 2, 49·32 ±1·84, P <0·001). When administered intraperitoneally to groups of adult male white mice (10 animals/group), these substances were well tolerated, even at a dose of 1·6 g/kg.

After examination of the compounds for the other pharmacological activities listed, it was apparent that no other action was worthy of note.

Discussion

The hypoglycaemic activity data for the arylsulphonylureas agreed well with those of Gerzon & others (1963). In view of this, and in the light of the results, it may be stated that in *N*-adamant-1-yl-*N*′-arylsulphonylureas, replacement of the 1-adamantyl group with a 1,1′-biadamantyl group leads to compounds having the same or an approximately doubled hypoglycaemic activity. If the pharmacological data are evaluated by the molar proportions instead of by weight, 3,3′-di(*N*′-*p*-toluenesulphonylureido)-1,1′-biadamantyl (Compd No. 1) and 3,3′-di(*N*′-*p*-methoxybenzenesulphonylureido)-1,1′-biadamantyl (Compd No. 3) are twice as potent as the corresponding 1-adamantyl derivatives while the less active 3,3′-di(*N*′-*p*-chlorobenzenesulphonylureido)-1,1′-biadamantyl (Compd No. 5) displays similar hypoglycaemic potency to the corresponding 1-adamantyl derivative. But the onset of action of the biadamantyl derivatives is distinctly delayed compared to that of the adamantane derivatives. Compounds Nos 1, 3 and 5 exert the maximum activity at the 6th hr, gradually and gently lowering the blood-glucose level while the corresponding adamantane derivatives act much more rapidly, reaching the maximum fall at the 2nd hr. This agrees with our expectations based on the fact that a greater molecular bulk would have been able to cause a marked reduction in the absorption rate.

Dimerization does not appear to influence the total duration of the hypoglycaemic action, which is almost the same for both series. Our substances increase the liver glycogen in the rat, as do the other well-known sulphonylureas. The most interesting compounds, Nos 1 and 3, are only slightly toxic, and apparently are free from any other noticeable pharmacological activity.

The almost complete lack of activity of 4,4′-di(*N*′-*p*-toluenesulphonylureido)bi(cyclohexyl) (Compd No. 7), particularly when compared with the high activity of the corresponding *N*-cyclohexyl-*N*′-*p*-toluenesulphonylurea(glycyclamide), indicates that the above monomer-dimer correlations must be limited, at present, in the sphere of the arylsulphonylureas, only to the *N*-adamant-1-yl-*N*′-arylsulphonylureas.

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The effect of antirheumatic drugs on the extractable collagen in lathyric chick embryos

K. TRNAVSKÝ AND Z. TRNAVSKÁ

The lathyrogenic effect of 2·5 mg of β-aminopropionitrile in the chick embryo has been measured by the increase in the extractability of collagen from bones using M saline. Sodium salicylate (3 mg/embryo) and hydrocortisone (1·5 mg/embryo) applied 24 hr after the injection of β-aminopropionitrile decreased the elevated amount of soluble collagen. Phenylbutazone, chloroquine diphosphate and sodium aurothiosulphate were ineffective in this experimental system.

THE lathyrogenic substances induce in experimental animals a generalized disease of connective tissue characterized by the occurrence of skeletal deformities, aneurysm and hernias (Selye & Bois, 1957). Underlying the disease is an increase in the amount of soluble collagen present in tissues, apparently resulting from decrease of covalent interchain cross-links (Levene & Gross, 1959; Martin, Piez & Lewis, 1963). Recently it has been postulated that lathyrogens may exert their effect by inhibition of the amine oxidase necessary for deamination of lysyl side-chains of collagen participating in cross-link formation (Page & Benditt, 1967).

Some substances from the group of hormones, antirheumatic drugs and divalent cations are able to influence favourably the lesions of experimental lathyrism as manifested by decrease of the elevated level of soluble collagen and the return of morphological changes to normal (Selye & Bois, 1957; Ponsetti, 1959; Trnavský, Trnavská & others, 1965; Naber, Scott & Johnson, 1965). These chronic experiments were made mostly in rats. To ascertain the influence of anti-rheumatic drugs in an acute experiment on a more closed system we used the assay in chicken embryos. We followed the method based on the increase of extractability of chick embryos bones in M cold saline after the injection of lathyrogen (Gross, Levene & Orloff, 1960).

Experimental

MATERIALS AND METHODS

Fertilized eggs of the white Leghorn variety were injected via the chorioallantoic membrane with 2·5 mg of β-aminopropionitrile fumarate in 0·1 ml of sterile distilled water at 14 days of incubation. After 24 hr the test drugs were administered in the same way and in the same volume. Control embryos were injected with 0·1 ml of distilled water on the 14th and 15th day of incubation. Each experimental group consisted of 30–35 embryos divided in 5 samples. The experiment was finished after another 24 hr when the tibiae and femora were stripped of adjacent tissues, minced and homogenized in the cold. A small sample was taken for the estimation of total hydroxyproline. The main part of the homogenate was then extracted in 2 volumes (v/w) of cold M saline containing phosphate (ionic strength 0·02, pH 7·6) for 24 hr with shaking in the cold (2°). After centrifugation the extraction was continued for a further 24 hr. The

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supernatants from centrifugation (1 hr at 30,000 *g*) were pooled and assessed by the method of Stegemann (1958) for the hydroxyproline content after hydrolysis with 6N hydrochloric acid.

The antirheumatic drugs tested were: sodium salicylate, hydrocortisone soluble (Spofa), phenylbutazone (Spofa), chloroquine diphosphate (Resochin-Bayer), Sanocrysin (sodium aurothiosulphate, Ferrosan).

Results

In the first experiment sodium salicylate at a dose of 3 mg depressed significantly the elevated amount of cold M saline-soluble hydroxyproline in β-aminopropionitrile-treated embryos. Salicylate itself did not change significantly the bone extractability. Hydrocortisone was also effective in decreasing the increased bone extractability of the nitrile-treated embryos. But in normal chicken embryos it decreased the content of soluble hydroxyproline. Phenylbutazone, 2 mg/embryo, chloroquine diphosphate, 5 mg/embryo, and Sanocrysin, 1 mg/embryo, were without effect.

TABLE 1. EFFECT OF ANTIRHEUMATIC DRUGS ON SOLUBLE HYDROXYPROLINE IN LATHYRIC CHICKEN EMBRYOS

Drug (dose/embryo)	Total hydroxyproline µg/100 mg of dry tissue	M NaCl-soluble hydroxyproline µg/100 mg of dry tissue	P
Control	1,362 ± 137	70 ± 12	
Sodium salicylate 5 mg	1,683 ± 62	71 ± 3	
β-Aminopropionitrile 2·5 mg	1,717 ± 51	348 ± 27	
β-Aminopropionitrile 2·5 mg + sodium salicylate 3 mg	1,677 ± 160	253 ± 34	<0·01
Control	1,390 ± 79	46 ± 8	
Hydrocortisone 1·5 mg	1,530 ± 84	36 ± 11	
β-Aminopropionitrile 2·5 mg	1,405 ± 54	230 ± 10	
β-Aminopropionitrile 2·5 mg + hydrocortisone 1·5 mg	1,342 ± 122	184 ± 18	<0·01
Control	1,556 ± 63	57 ± 5	
Phenylbutazone 3 mg	1,932 ± 212	73 ± 4	
β-Aminopropionitrile 2·5 mg	1,861 ± 66	201 ± 13	
β-Aminopropionitrile 2·5 mg + phenylbutazone 2 mg	1,864 ± 11	202 ± 24	
Control	1,859 ± 77	61 ± 4	
Chloroquine 5 mg	1,796 ± 26	66 ± 8	
β-Aminopropionitrile 2·5 mg	1,775 ± 45	265 ± 22	
β-Aminopropionitrile 2·5 mg + chloroquine 5 mg	1,859 ± 125	290 ± 24	
Control	2,102 ± 143	62 ± 11	
Sanocrysin 1 mg	2,229 ± 208	81 ± 7	
β-Aminopropionitrile 2·5 mg	1,846 ± 266	331 ± 19	
β-Aminopropionitrile 2·5 mg + Sanocrysine 1 mg	1,914 ± 196	362 ± 25	

Discussion

Sodium salicylate and hydrocortisone proved to be effective in depressing the increased solubility of lathyric collagen. But at the time when the tested drugs were injected, β-aminopropionitrile had been acting for 24 hr, so we would not expect the M sodium chloride soluble hydroxyproline level to completely return to normal. Most of the antirheumatic drugs return increased levels of skin-soluble hydroxyproline in lathyric

rats to normal. Even in this acute experiment in chicken embryos, which differs from the chronic experiment in rats, two of the drugs were effective.

The mechanism of action of antirheumatic drugs in experimental lathyrism is difficult to explain. Hydrocortisone is known to decrease the content of soluble collagen fractions in tissues and to promote the conversion of soluble into insoluble collagen (Kivirikko, 1963; Kühn, Iwangoff & others, 1964). The combined inhibitory effect on biosynthesis and at the same time promotion of collagen maturation could be responsible for the influence of hydrocortisone in experimental lathyrism. Knowledge about the influence of salicylate on the metabolism of collagen is scarce. Preliminary observations have shown that sodium salicylate is capable of promoting the transformation of soluble into insoluble collagen (Trnavská, Trnavský & Kühn, 1968). The influence of antirheumatic drugs on the collagen defect in experimental lathyrism is probably a result of a more general metabolic effect. Whether the ameliorating effect of antirheumatic drugs, at least in chronic experiments, is a characteristic feature for this group of drugs remains to be solved.

This report provides further evidence that it may be possible to modify the lathyric changes. Besides hormones, divalent cations and antirheumatic drugs are able to influence the lathyric toxicity favourably to some extent.

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Interaction of aspirin with nonsteroidal anti-inflammatory drugs in rats

Sir,—Clinical evaluation of new anti-arthritic drugs is difficult because of the unpredictable course of arthritis. It is even more difficult if an analgesic drug, such as a salicylate which also has anti-inflammatory activity (Smith & Smith, 1966), is administered at the same time. The difficulty in assessing the anti-inflammatory activity of a nonsteroidal drug, while a mild analgesic (usually a salicylate) is permitted *ad libitum*, is reflected in some of the reports on the anti-arthritic activity of indomethacin. While numerous investigators have reported indomethacin to be active against arthritis, Mainland (1967) and Donnelly, Lloyd & Campbell (1967) concluded that indomethacin was approximately as effective as placebo. Of the reports in which indomethacin was found to be active, only a few stated whether salicylates were permitted. In three of these, indomethacin was compared side-by-side with a salicylate (Pinals & Frank, 1967; Gaspardy, Gaspardy & others, 1966; Pitkeathly, Banerjee & others, 1966); in two other reports salicylates were gradually withdrawn in some cases (Rothermich, 1966; Englund, 1966); and in one study, free intake of aspirin was allowed (Smyth, 1965).

Does the intake of salicylates mask the anti-inflammatory activity of anti-inflammatory agents? In an attempt to answer this, two anti-inflammatory drugs, indomethacin and phenylbutazone, have been administered alone or in a combination with aspirin to rats in which oedema in the foot had been induced by carrageenan according to a slightly modified method of Winter, Risley & Nuss (1962).

Male Sprague-Dawley rats from A and E Farms, Altamont, N.Y., 125 g, were fasted overnight and indomethacin (Merck), 1·56 and 6·25 mg/kg, and phenylbutazone (Geigy), 25 and 100 mg/kg, were administered by stomach tube as suspensions in 1% gum tragacanth, 2 ml/100 g. Aspirin (Monsanto) was administered similarly at 100 mg/kg alone and at 100 mg/kg in combinations with each of the above doses of indomethacin or phenylbutazone. Control rats received only the gum tragacanth vehicle. Each single and combined dose was examined in duplicate experiments.

One hr after administration of the drugs, 0·05 ml of 0·75% carrageenan (Viscarin standard) suspension was injected into the plantar tissue of the right hind foot of each rat. Three hr later, all rats were killed with chloroform and both feet were cut off at the tibio-talar joint, and weighed. The oedema was determined from the difference between weights of injected and non-injected feet, and the percent inhibition of oedema was calculated for each group. The significance of difference between the medicated groups and the control groups, and between the groups that received combined drugs and single drugs were calculated according to the *t*-test.

The doses of all compounds were selected so that, in the event of additive inhibition of oedema, the maximal inhibition due to combined medication would not be expected to be greater than the limits of attainable inhibition in this test system.

Table 1 shows that aspirin, 100 mg/kg, or indomethacin or phenylbutazone at two doses each resulted in significant ($P \leq 0·01$) inhibition of oedema in all experiments. The degree of inhibition by indomethacin or phenylbutazone was dose-related.

Combined administration of aspirin with indomethacin resulted in inhibition of oedema that was not significantly different from that obtained with the same doses of either compound alone.

Combined administration of aspirin with phenylbutazone resulted in slightly

TABLE 1. INHIBITION OF CARRAGEENAN OEDEMA BY COMBINED ADMINISTRATION OF ASPIRIN WITH INDOMETHACIN OR PHENYLBUTAZONE

	Aspirin (mg/kg)							
	0		100		0		100	
	Oedema (mg)	Inhibition (%)	Oedema (mg)	Inhibition (%)	Oedema (mg)	Inhibition (%)	Oedema (mg)	Inhibition (%)
Indomethacin (mg/kg)	Exp. 1 (5 rats/group)				Exp. 2 (8 rats/group)			
0	647 ± 25	—	349 ± 44	46	556 ± 27	—	343 ± 35	38
1·56	402 ± 26	38	526 ± 18	34	329 ± 20	41	394 ± 13	29
6·25	320 ± 65	50	294 ± 37	54	271 ± 26	51	290 ± 39	48
Phenylbutazone (mg/kg)	Exp. 1 (10 rats/group)				Exp. 2 (10 rats/group)			
0	647 ± 25	—	349 ± 44	46	648 ± 30	—	381 ± 29	41
25	386 ± 29	40	273 ± 38	58*	364 ± 22	44	247 ± 42	62*†
100	302 ± 26	53	371 ± 55	43	279 ± 32	57	207 ± 42	68†

* $P < 0{\cdot}05$ for differences between inhibition due to combined drugs and aspirin alone.
† $P < 0{\cdot}01$ for differences between inhibition due to combined drugs and phenylbutazone alone.

greater inhibition of oedema than that achieved with aspirin alone in one of the experiments at the lower dose of phenylbutazone and this increase was significant at the $P \leq 0{\cdot}01$ level, but no significant differences were noted in the other experiment or at the higher dose.

In comparing the effects of administering aspirin with phenylbutazone with those of phenylbutazone alone, aspirin with 25 mg/kg of phenylbutazone resulted in slightly greater inhibition ($P \leq 0{\cdot}05$) than that of 25 mg/kg of phenylbutazone alone in both experiments, but combined administration of aspirin with 100 mg/kg of phenylbutazone resulted in no significant differences compared to administration of 100 mg/kg of phenylbutazone alone.

All rats were normal in behaviour and appearance throughout.

The results of the combined medications of aspirin and indomethacin in rats indirectly support the conclusions of Mainland (1967) and Donnelly & others (1967) who reported that the effects of indomethacin (in patients receiving salicylates *ad libitum*) were indistinguishable from the effects of placebo treatment. The combined administration of aspirin with indomethacin to rats resulted in no additive inhibition of oedema which suggests drug interaction.

The results after combined administration of aspirin with phenylbutazone were not as consistent, but even here the anti-inflammatory responses were not strictly additive, and they again suggest drug interaction.

These data lend support to the claim by Boardman & Hart (1967) that high doses of salicylates are anti-inflammatory as well as analgesic, and that the intake of salicylates should be eliminated from comparative clinical trials of non-steroidal anti-inflammatory agents.

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The reactivity of the pregnant rat myometrium

SIR,—It has been demonstrated (Schofield, 1957) that the actomyosin concentration of the rabbit myometrium, measured by the tension developed to optimal electrical stimulation, increases from mid-term to parturition and that the synthesis of actomyosin is regulated by oestrogens (Csapo, 1950). For the greater part of pregnancy, the rabbit myometrium is progesterone-dominated and refractory to oxytocin (Schofield, 1957). A possible mechanism by which progesterone exerts its "blocking" action may be due to the greater effectiveness of calcium binding which the progestrone-dominated uterus shows when compared with the oestrogen-dominated uterus (Csapo, 1961). The present work was undertaken to assess the influence of the female sex hormones on the pregnant rat myometrium.

Wistar rats 13–15 weeks old were with males for 4 days and subsequently separated into groups at different periods of gestation; each group therefore represented a 4 day period of gestation as shown in Table 1. Those animals used on the day of parturition were killed within 6 hr of completion of delivery and further animals were used for 3 days post-partum. A group of 10 non-pregnant animals served as controls.

The animals were stunned, decapitated, and the uteri rapidly dissected into a dish of modified Krebs solution (Knifton, 1966) at 4°. One uterine horn from each animal was incised longitudinally and the foetuses removed. A strip of uterus 25 mm × 7 mm was cut, transferred to a 10 ml tissue bath and assembled for electrical stimulation and isometric recording as previously described (Knifton, 1966).

All experiments were made with the tissues adjusted to resting length. After a 30 min resting period to allow the pattern of spontaneous motility to become apparent, the minimum dose of oxytocin (Syntocinon, Sandoz) which caused a uterine contraction (oxytocin threshold) was determined. The tissue was then stimulated electrically at 1 min intervals at optimum voltage, each stimulus of 5 sec duration. When the contractions attained a steady state tension, the tissue was washed repeatedly in calcium-free Krebs solution and the time when the tension was reduced to 50% of the steady state tension (T50) was measured.

The results are summarized in Table 1.

The values for steady state tension increased as gestation advanced to reach a maximum at the time of parturition. When the mean steady state tension of each group is compared with that of the controls however, the difference in tension is not significant until the 17–20 day period of gestation. That is, the steady state

TABLE 1. THE RESPONSE OF THE PREGNANT AND POSTPARTUM RAT UTERUS TO ELECTRICAL STIMULATION AND OXYTOCIN. THE FIGURES SHOW THE MEAN VALUE (±S.E.) FOR EACH GROUP OF ANIMALS.

		Stage of gestation (days)					Stage post-partum (days)			
	Controls	1–4	5–8	9–12	13–16	17–20	Partn	P + 1	P + 2	P + 3
Steady state tension (g) ..	4·11 ±0·65	3·70 ±0·71	3·73 ±0·67	4·35 ±1·02	5·72 ±1·12	6·40 ±1·04	8·55 ±1·21	8·72 ±1·09	4·78 ±0·89	3·58 ±0·43
T50 (min)	4·05 ±0·77	3·55 ±0·95	4·73 ±1·07	6·77 ±1·59	7·03 ±1·23	6·70 ±1·67	1·58 ±0·28	3·01 ±0·50	3·43 ±0·64	3·74 ±0·77
Oxytocin threshold (mU)	0·91 ±0·51	0·57 ±0·42	1·54 ±0·33	3·00 ±1·16	2·34 ±1·17	0·98 ±0·39	0·31 ±0·17	1·14 ±0·45	0·53 ±0·25	1·16 ±0·41
Number of animals per group ..	10	7	8	8	9	13	11	13	10	12

tension in the 17–20 day group, the animals on the day of parturition and those the day after parturition is significantly greater ($P < 0{\cdot}05$) than that of the controls. Two days after parturition, tension had fallen abruptly. It has been shown (Csapo & Corner, 1953) that, other factors being equal, the tension developed by the myometrium to optimal electrical stimulation is proportional to the actomyosin concentration, which reflects the degree of influence of oestrogens. This would indicate that in the rat, there is a fairly sudden rise in oestrogen output towards the end of gestation to reach a peak at parturition and an abrupt post-partum decline. The conclusion is endorsed by other work (Knifton, 1967) where it was shown that, in the rat, progesterone does not affect the tension developed by the myometrium to optimal electrical stimulation.

When compared with the non-pregnant controls, the sensitivity to oxytocin did not change significantly throughout gestation until the day of parturition when it increased ($P < 0{\cdot}05$). There is also a difference in sensitivity to oxytocin ($P < 0{\cdot}05$) between the parturient group and the group in mid pregnancy (9–12 days).

The degree of calcium binding in the myometrium, indicated by the time taken to reduce tension by 50% in calcium-free Krebs solution (T50), approximates to the oxytocin sensitivity at different stages of pregnancy. Calcium is bound most effectively during mid pregnancy; the difference between the 13–16 day group and the controls is significant at the 5% level. There is an abrupt decrease in calcium binding on the day of parturition which coincides with the sudden increase in sensitivity to oxytocin. These findings are in accord with the evidence reviewed by Daniel (1964) that when calcium is effectively bound in smooth muscle membrane, the tissue is relatively refractory to stimulation.

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Species differences in the plasma protein binding of desipramine

SIR,—Species differences have been demonstrated for the plasma protein binding of acidic drugs (Anton, 1960; Sturman & Smith, 1967), but to our knowledge such information is not available for basic drugs. It is generally accepted that only the unbound portion of a drug is available for pharmacological activity. The unbound portion also is available to the liver for metabolism and appears in the ultrafiltrate in the kidney glomeruli. Hence, the degree of protein binding can be of great quantitative significance in the pharmacological (therapeutic as well as toxic) action of drugs. Marked species and strain differences have been reported for the pharmacological effects of one group of basic drugs, the tricyclic antidepressants (Brodie, 1965). The work now reported was undertaken to determine the degree of plasma protein binding of desipramine in various species.

Blood from the various species was collected in tubes containing heparin sodium and centrifuged at 2,000 rev/min. If analyses were not made the same day, the plasma was stored at 4° for 15–24 hr. The plasma was incubated at room temperature (21–24°) with [^{3}H] desipramine (1·1 μM, 14·7 mc/mmole, chromatographically pure) for 60 min. The degree of protein binding was determined by ultrafiltration at room temperature (Schanker & Morrison, 1965). [^{3}H]Desipramine was determined in the ultrafiltrate and in the plasma by extraction of an alkalinized aliquot with the toluene scintillation mixture. Appropriate corrections were made for the minimal quenching found in the samples.

The species differences in the plasma protein binding of desipramine are as follows.

Species	Unbound desipramine %
Man	8·6, 10·5, 10·0, 10·8, 9·0
Rat	10·6, 9·9, 6·0, 6·0, 7·4
Rabbit	13·8, (14·2), 16·1, 15·3, 16·4, 15·9
Dog	2·0, 4·0, 0·7, 3·8, 4·0
Cat	1·5, 1·8, 2·1, 3·2

Each value is the mean of at least two duplicate determinations of individual plasma samples for all species except the rat where the values represent results from a pool of 4–6 rats. The values were determined at a desipramine concentration of 1·1 μM. The value of 14·2 was obtained from plasma from a rabbit 1 hr after intravenously administered desipramine, 10 mg/kg. The desipramine was determined by the method of Hammer & Brodie, 1967. The *in vitro* and *in vivo* values correlate very well.

It is interesting to speculate whether the reported species differences in pharmacological effect of tricyclic antidepressants could be related to the degree of protein binding.

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The dual mode of action of histamine in the cat isolated tracheal chain

SIR,—In the cat isolated tracheal chain, a fully relaxed preparation *in vitro* (Hawkins & Paton, 1944), histamine antagonizes non-specifically the contractions induced by acetylcholine (Akcasu, 1952) and potassium chloride (Akcasu, 1959). Since in the isolated heart histamine effects have been attributed to a direct interaction with mycocardial receptors (Mannaioni, 1960), or to an indirect action based on the release of catecholamines (Went, Szucs, & Feher, 1954), it is of value to establish the mechanism of histamine relaxation of cat tracheal chains.

Tracheal rings of cats, untreated or pretreated with reserpine (0·5 mg/kg, injected intraperitoneally 48 and 24 hr before the experiment), were prepared according to the modification by Akcasu (1959) of the method described by McDougal & West (1953), and studied in Tyrode solution aerated with oxygen 95% and carbon dioxide 5% at 37 ± 0·5°. A dose of carbamylcholine chloride (carbachol), chosen from dose-response curves to produce a 60 to 85% of maximum contraction in a 2-ring chain, was left in contact with the tissue for 6·5 min. Histamine dihydrochloride was added for the last 1·5 min of this time. When antagonists were used they were added with the carbachol. All doses are expressed as μg of salt per 30 ml of bath volume. Responses, magnified 16 times, were recorded on a smoked drum kymograph with an auxotonic pendulum lever (Paton, 1957). Statistical calculations were made according to Snedecor (1957).

Only 7 of the 19 preparations taken from untreated cats showed a relaxation to 50 μg of histamine while all relaxed to 100 μg (Table 1). The relaxation was dose-dependent and reached a maximum with 200 μg of histamine. The relaxing action of histamine was only partially prevented by mepyramine maleate (40 μg). Similarly pronethalol (40 μg), a dose which fully blocked the relaxing effect of l-(–)-noradrenaline bitartrate (2·5 to 10 μg) was only partially effective against histamine. Neither inhibitor, in the concentrations stated, altered

TABLE 1. DEPRESSION IN MM BY HISTAMINE (H) OF CARBACHOL[1]-CONTRACTIONS[2] OF CAT TRACHEAL RINGS.

H-Dose: μg/30 ml	50	100	200	400	800	1600	Slope ± s.e.
Untreated							
Mean ± s.e. (n)	−19·3 ± 4·16 (7)	−28·2 ± 4·97 (19)	−43·5 ± 6·48 (19)	−44·2 ± 6·79 (13)			30·35 ±10·81
Reserpine-pretreated							
Mean ± s.e. (n)				−9·7 ± 2·06 (6)	−18·3 ± 2·97 (7)	−33·0 ± 5·24 (7)	39·35 ± 8·96

(1) Mean dose: 1·41 ± 0·081 μg/30 ml (2) Mean contraction height: 92·7 ± 3·45 mm

contraction heights to carbachol, but higher concentrations of either inhibitor reduced them. When both inhibitors were added simultaneously (40 μg of each) histamine relaxations were fully counteracted, but carbachol contractions were partly suppressed.

Sensitivity to carbachol was unaffected in tracheal rings taken from reserpine-pretreated cats. Despite the catecholamine depletion, histamine relaxed these preparations, but the doses required were much higher (Table 1), the threshold dose of histamine being 400 μg while the response to 800 μg was about equivalent to that produced by 50 μg in tracheal rings from untreated cats. The addition of 3200 μg of histamine to two of the preparations increased the relaxations further, to -53 mm. The slopes of the dose-response curves to histamine in tracheal chains from untreated and reserpine-pretreated cats were parallel, but their x-intercepts were significantly different ($P < 0{\cdot}001$); the x-intercept for the untreated preparations was 10·49 μg, whereas it was 244·4 μg for the catecholamine-depleted tracheal rings. Exposure of the catecholamine-depleted tracheal chains to 10 μg of noradrenaline bitartrate for 10 min, followed by a 5-min washing period, approximately doubled the relaxing potency of histamine.

The observation that mepyramine and pronethalol partly counteracted relaxations to histamine in tracheal rings from untreated cats, together with the ability of histamine to relax catecholamine-depleted tracheae, permit the conclusion that histamine has a dual mode of action in this preparation: (1) it releases catecholamines which stimulate adrenergic β-receptors to induce relaxation and (2) it combines with its own specific receptors to induce relaxation.

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An interaction between desipramine and phenylbutazone

SIR,—Imipramine and other tricyclic antidepressant agents are known to be inhibitors of liver microsomal enzymes (Kato, Chiesara & Vassanelli, 1963). I now report a novel effect of desipramine on the intestinal absorption of phenylbutazone.

Female, Sprague–Dawley rats, 150 g, were injected with desipramine (3·75–15 mg/kg i.p.), and 1 hr later phenylbutazone was given either intraperitoneally (20 mg/kg) or orally (40 mg/kg).

At specified times, the rats were decapitated and the blood collected in centrifuge tubes containing 0·1 ml of 2% heparin. After centrifugation, the phenylbutazone and oxyphenylbutazone were measured in the plasma (Herrmann, 1959).

Desipramine had no effect on the rate of decline of plasma levels of phenylbutazone during 4 hr, and only a small effect on these levels after 6 hr, when the phenylbutazone was administered intraperitoneally (Table 1). However, when phenylbutazone was given orally, pretreatment with desipramine or imipramine (15 mg/kg, i.p.) decreased the level of phenylbutazone in the blood plasma (Table 2).

A dose of 3·75 mg/kg of desipramine reduced the plasma concentration of phenylbutazone (40 mg/kg orally 1 hr later) by 45% from the control value (95 ± 3·5 to 53 ± 5 μg/ml; $P < 0·01$), while a dose of 15 mg/kg of desipramine lowered the plasma level of phenylbutazone by 82% (to 17 ± 3 μg/ml; $P < 0·01$). Phenylbutazone was measured 2 hr after oral dosage. The decreased plasma levels of orally administered phenylbutazone after desipramine pretreatment were not due to an increased metabolism of phenylbutazone since

TABLE 1. PLASMA LEVELS OF PHENYLBUTAZONE (I.P.) IN CONTROL AND IN DESIPRAMINE PRETREATED RATS

Time (hr) after phenylbutazone (20 mg/kg i.p.)	Plasma phenylbutazone (μg/ml) ± s.e.	
	Saline	Desipramine (10 mg/kg i.p.) 1 hr before phenylbutazone
1	85·7 ± 6·3	87·5 ± 7·3
2	71·3 ± 3·6	67·3 ± 3·8
3	57·5 ± 2·6	59·9 ± 3·6
4	54·7 ± 3·7	52·8 ± 3·7
6	34·7 ± 3·0	*49·5 ± 2·9
7	34·2 ± 3·4	43·3 ± 5·3
8	21·0 ± 2·4	33·8 ± 2·4

* = $P < 0·01$

TABLE 2. PLASMA LEVELS OF PHENYLBUTAZONE (ORAL) IN CONTROL AND IN DESIPRAMINE OR IMIPRAMINE PRETREATED RATS

Time after phenylbutazone (40 mg/kg oral)	Plasma phenylbutazone (μg/ml) ± s.e.		
	Saline	Imipramine (15 mg/kg, i.p.)†	Desipramine (15 mg/kg, i.p.)†
20 min	28·0 ± 3·4	25·1 ± 3·6	**15·2 ± 2·6
1 hr	109·6 ± 5·8	*40·9 ± 4·3	*28·5 ± 3·1
2 hr	98·7 ± 3·0	*31·6 ± 5·4	*33·6 ± 3·8
4 hr	87·9 ± 5·3	*47·2 ± 3·5	*50·4 ± 6·8

† = Given 1 hr. before phenylbutazone.
* = $P < 0·01$. ** = $P < 0·05$.

oxyphenylbutazone levels were also lowered by pretreatment with desipramine from a control value of 22 ± 5 μg/ml to 2 ± 1·5 μg/ml at a dose of desipramine of 15 mg/kg indicating a possible effect of desipramine on phenylbutazone metabolism. This inhibitory effect on metabolism was also noticed after 6 hr when phenylbutazone was given intraperitoneally (Table 1).

These findings suggest desipramine interferes with the intestinal absorption of phenylbutazone. The effect of desipramine on the metabolism of phenylbutazone appears to be important in the light of the high doses required and the minor influence on the phenylbutazone and oxyphenylbutazone blood levels.

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Guanethidine and carbachol on the isolated frog rectus: a non-competitive interaction

SIR,—Guanethidine has been found to inhibit contractions caused by direct nerve and by direct muscle stimulation (Dixit, Gulati & Gokhale, 1961; Kroneberg & Stoepel, 1962; Green & Hughes, 1966; Chang, Chen & Cheng, 1967). Its main effect appeared to be on muscle fibres (Chang & others, 1967). On the other hand, in experiments in which avian and frog muscle were used, Rand & Wilson (1967b) concluded that guanethidine was a competitive antagonist of acetylcholine in these preparations. Gokhale, Gulati & others (1963), Chang & others (1967) and Rand & Wilson (1967b) attempted to modify the responses of the frog rectus to single doses of acetylcholine, but failed to analyse the dose-response curves before and after exposure to guanethidine. In recent work, Feinstein & Paimre (1967) used the same preparation to compare the effects which the competitive antagonist (+)-tubocurarine and the non-competitive antagonist tetracaine exerted on contractions elicited by carbachol, and found that while the first drug produced a parallel shift to the right of the dose-response curves, the second mainly reduced maximum contractility.

The object of the present work was to find out whether guanethidine was a competitive or non-competitive inhibitor of carbachol.

In Ringer solution, with oxygen bubbled through it, contractions of isolated rectus abdominis muscle of *Rana esculenta* were elicited by carbachol, of which the end-concentrations were $0{\cdot}2 \times 10^{-6}$ g/ml and the 2, 4, 8, 16-fold of it. The carbachol concentration was always doubled, without washing, when the effect of the preceding concentration had fully developed, and the dosing was continued until the maximum contraction developed. Five and 10 min after addition of the antagonists tubocurarine and guanethidine, respectively, the above procedure was repeated.

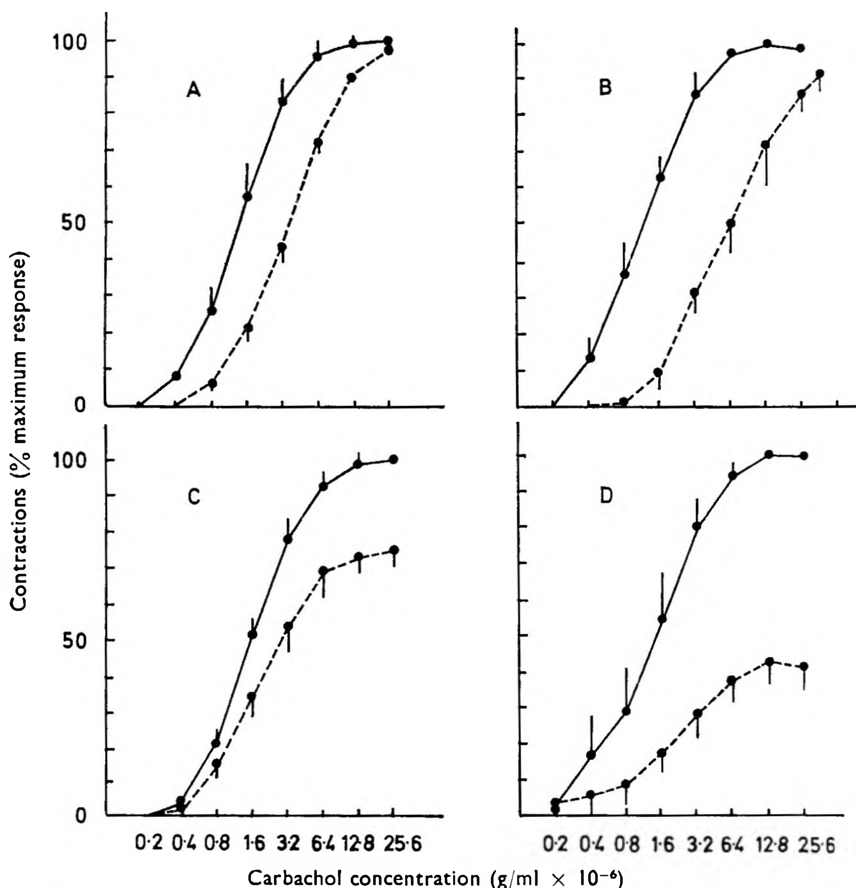

FIG. 1. Carbachol dose-response curves before and after the antagonists on frog isolated rectus. Each point is the mean $\pm$ the standard error. A. 2×10^{-7} g/ml tubocurarine, mean values of 6 experiments. pA_2 6·72 $\pm$ 0·26. B. 5×10^{-7} g/ml tubocurarine, mean values of 5 experiments. pA_2 6·79 $\pm$ 0·36. C. 5×10^{-6} g/ml guanethidine, mean values of 7 experiments. pD_2 4·19 $\pm$ 0·32. D. 10^{-5} g/ml guanethidine, mean values of 6 experiments. pD_2 4·55 $\pm$ 0·28. ($\pm$ values: probability interval, P_{95}, for the mean values, Ariëns & Simonis, 1961).

In our experiments the two antagonists could be washed out; on the same preparation the carbachol effect could be inhibited and restored 3 or 4 times.

The results illustrated in Fig. 1 show that guanethidine produces no parallel shift to the right of the dose-response curves for carbachol, but depresses the maximum; it acts in the way of tetracaine and, therefore, cannot be considered a competitive antagonist.

It is not impossible that the non-competitive antagonistic effect of guanethidine on carbachol stems from its local anaesthetic property. Adrenergic neuron blocking agents and classical local anaesthetics differ essentially in their activities, for example, the latter drugs act quickly and can be readily washed out (Rand & Wilson, 1967a), but this is not evidence for a difference in the mechanism of action. The classical local anaesthetics are lipophilic and the

adrenergic blocking agents lipophobic. Both the quaternary local anaesthetics (Herr, Nádor & others, 1953) and the adrenergic neuron blocking agents are characterized by their slow but persistent action. The relation between local anaesthetic and adrenergic neuron blocking activity was first observed by Hey & Willey (1954). The present work seems to confirm the statement of Boura & Green (1965): "the possibility remains that the depressant action of the adrenergic fibre terminals is analogous to the impairment of nerve conduction in nerve trunks caused by local anaesthetics".

Our results show that on the rectus abdominis muscle of the frog, guanethidine like the local anaesthetics, is a non-competitive antagonist to carbachol.

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The effect of diethyldithiocarbamate on brain amine levels in the rabbit

Sir,—Sodium diethyldithiocarbamate or its oxidation product disulfiram inhibit both dopamine β-hydroxylase and monoamine oxidase in the brains of rats or guinea-pigs (Yamada & Yasunobu, 1962; Goldstein & Contrera, 1961; Musacchio, Kopin & Snyder, 1964; Collins, 1965; Carlsson, Lindquist & others, 1966). While investigating the neurotoxic action of sodium diethyldithiocarbamate in the rabbit (Edington, 1967), I have found differences in the level of central nervous system amines in this animal.

Twelve adult male or female Dutch rabbits 1·8–2·4 kg, were paired in similar weights and given sodium diethyldithiocarbamate as a buffered isotonic solution (Sunderman, White & others, 1963) at 750 mg/kg or saline intravenously. Two hr after the injection the rabbits were killed in a cold room, the brain removed, sectioned sagitally, and one half placed in a preweighed homogenized tube containing 10·0 ml of ice cold acid butanol. This tissue was homogenized and subsequently diluted to 30·0 ml. Fluorimetric estimations of 5-hydroxytryptamine (5-HT) and noradrenaline were made on the homogenized sample. The treated animals (6) had an estimated 5-HT brain content of 0·67 $\pm$ 0·08 μg/g brain while the controls (6) had 0·45 $\pm$ 0·07 μg/g wet weight. Noradrenaline

(0·68 ± 0·11 μg/g wet weight) was only detectable in the controls. The monoamine oxidase activity of the other half of the brain was measured using the method of Green & Haughton (1961). The treated animals (6) had a monoamine oxidase activity (measured as extinction) of 0·07 ± 0·03 while the controls (6) registered 0·25 ± 0·14. Two hr after the administration of sodium diethyldithiocarbamate, monoamine oxidase activity in the CNS was severely inhibited, 5-HT levels were raised, and noradrenaline levels were depressed. Carlsson & others (1966), measured noradrenaline and dopamine levels biochemically in rats and showed that noradrenaline levels were depleted while whole brain dopamine levels were not altered by sodium diethyldithiocarbamate, nor was it found by histochemical techniques to cause detectable changes in noradrenaline, dopamine or 5-HT. The results in rabbits confirm the depression of noradrenaline levels in the brain by sodium diethyldithiocarbamate. They also indicate that there is a significant increase in 5-HT levels when measured biochemically, and this is presumably due to the decrease in activity of monoamine oxidase. This increase in 5-HT may well have been more pronounced in rabbits due to the larger dose (750 mg/kg compared with 500 mg/kg used in rats by Carlsson & others) and the more direct route of administration (intravenous as opposed to subcutaneous). That this is so is supported by the fact that in the rabbits used in the present work noradrenaline could not be detected biochemically after the intravenous administration of sodium diethyldithiocarbamate, whereas Carlsson & others recorded only a 70% depression in levels after a single subcutaneous injection and a 90% depression after a second subcutaneous injection. The action of sodium diethyldithiocarbamate on CNS amines was of particular interest as I have shown that, given by injection over a period of months, it produces a neuraxonal dystrophy in rabbits and in hens. Whether the neuropathological lesion and the changes in brain amine levels are related, remains speculative since it is also known that sodium diethyldithiocarbamate inhibits a wide range of enzymes (Thorne & Ludwig, 1962) and also produces abnormally high levels of copper in the CNS and liver when given repeatedly (Edington & Howell, 1966).

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May 14, 1968

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Drug-induced rhythmicity in smooth muscle

SIR,—When isolated in organ-bath experiments, the vas deferens of the guinea-pig or rat is usually quiescent, but several workers have reported the initiation of rhythmical contractions by drugs (Birmingham & Wilson, 1963; Boyd, Burnstock & others, 1963; Ohlin & Strömblad, 1963; Burnstock & Holman, 1964; Bentley, 1965, 1966). In the experiments described here, cocaine, procaine and lignocaine induced reproducible contractile activity.

Adult guinea-pigs (400–750 g) and rats (200–300 g) were killed by stunning and bleeding, and both vasa deferentia were removed. These were suspended in Krebs solution at 32°, oxygenated with 95% oxygen and 5% carbon dioxide. Recording of contractions was with frontal writing levers on smoked paper, using a 600 mg load and 5 × magnification.

Test vasa were exposed to increasing concentrations of drug, and it was noted that activity was initiated at a threshold concentration (Table 1). This activity consisted of rhythmical contractions and relaxations (Fig. 1), and with each successive increase in concentration, an increase in amplitude or frequency,

TABLE 1. INITIATION OF RHYTHMICAL ACTIVITY. ACTIVITY THRESHOLD AND OPTIMUM CONCENTRATIONS (MOLAR) OF COCAINE, PROCAINE AND LIGNOCAINE

	Guinea-pig				Rat			
	Normal		Denervated		Normal		Denervated	
	Threshold	Optimum	Threshold	Optimum	Threshold	Optimum	Threshold	Optimum
Cocaine..	1×10^{-4}	1×10^{-3}	5×10^{-4}	1×10^{-3}	1×10^{-5}	1×10^{-3}	1×10^{-4}	1×10^{-3}
Procaine	1×10^{-3}	1×10^{-2}	1×10^{-3}	1×10^{-2}	5×10^{-4}	5×10^{-3}	1×10^{-4}	1×10^{-2}
Ligno-caine ..	2×10^{-4}	5×10^{-3}	1×10^{-3}	5×10^{-3}	1×10^{-4}	1×10^{-3}	5×10^{-6}	1×10^{-3}

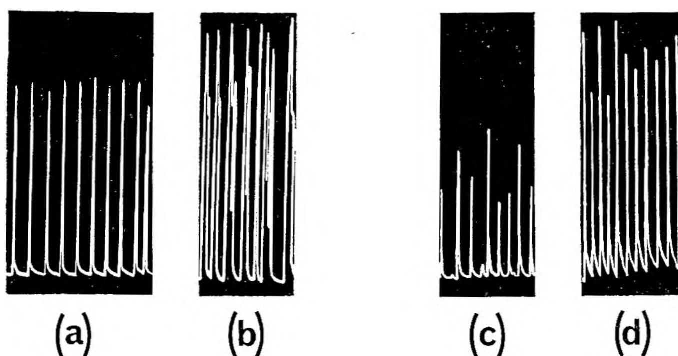

FIG. 1. Response of the guinea-pig vas deferens to local anaesthetics. (a) Rhythmical contractions produced by the guinea-pig vas deferens immersed in 5×10^{-3}M lignocaine. Such activity was often seen to persist for many hours. (b) Rhythmical contractions produced in 2×10^{-3}M procaine. The multiphasic contractions were often seen when high concentrations of cocaine, procaine and lignocaine were used. (c) Activity produced in response to 1×10^{-3}M lignocaine. This specimen had intact intramural nerves at the time of removal. (d) The denervated partner of the specimen in (c) produced considerably more activity in 1×10^{-3}M lignocaine. The denervation was of 8 days' duration.

or both, was noted. The notable feature of the responses obtained after the application of drug was their amplitude and frequency, and their similarity to those obtained from the electrically stimulated vas, (Fig. 1a). An optimum drug concentration was usually obtained, after which the activity rapidly declined. Activity could be terminated at any point by washing out the drug with normal Krebs solution, and could be immediately restituted by the return of the drug. It may therefore be assumed that the contractions were initiated by the drug.

A control vas, not exposed to drug, remained inactive throughout the duration of each experiment. Generally speaking, the vas deferens of the rat appeared to be the more sensitive for the threshold and optimum drug concentrations, although the actual contractions were smaller than those of the guinea-pig.

On the basis of the amount of activity, the most potent agent tried appeared to be procaine (Fig. 1b), but several other drugs also produced a great deal of activity. These were piperoxan, thymoxamine and mepyramine. The ability to produce rhythmical activity, and the local anaesthetic properties do not seem to be related, since some agents which produced potent local anaesthesia were unable to stimulate the tissue. For example, benzocaine, chlorcyclizine and chlorpromazine were ineffective.

Is the phenomenon dependant on the nerve-supply to the tissue? That this does not seem to be so is indicated by experiments made on surgically denervated tissue.

Denervated vasa were obtained from guinea-pigs and rats which had one vas denervated eight days previously (Birmingham, 1967, 1968). When the denervated vas was set up alongside its innervated partner from the same animal, both became rhythmically active when exposed to drug. The denervated vas was usually more active at its threshold and optimum drug concentrations than its control. These results indicate that an intact intramural nervous system in the tissue is not essential for the initiation of the type of activity described.

In addition, vasa from animals pretreated with reserpine were still able to achieve as much activity as their controls. That catecholamines are not necessary for this activity was indicated by histochemical studies using a modified Falck's fluorescence technique (Spriggs, Lever & others, 1966), which showed complete depletion of fluorescent nerve-fibres in the tissue after reserpine treatment.

It seems legitimate to conclude that drugs like procaine, cocaine and lignocaine which activate the tissue in the manner described, do so by an action on the smooth muscle cells which is independent of their innervation.

Acknowledgement. The author wishes to thank Dr. A. T. Birmingham for supplying the denervated and reserpinized animal specimens, and the Medical Research Council for the Scholarship which enabled this work to be carried out.

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Enhanced toxicity of imipramine and desipramine in aggregated mice

SIR,—From experiments with amphetamine (Cohen & Lal, 1964) and cocaine (Lal & Chessick, 1965), it was postulated that the enhanced toxicity of these drugs in aggregated mice was related to their common property by which they inhibit tissue uptake of catecholamines. Recently, imipramine and desipramine were found to block tissue uptake of noradrenaline *in vitro* (Iversen, 1965) and *in vivo* (Glowinski & Axelrod, 1964). The present work shows that aggregation of mice enhanced the toxicity of both drugs.

Swiss albino random-bred male mice of 22–28 g were placed in stainless steel cages (7 × 9·5 × 7 inches), 10 to a cage, 2 hr or more before the intraperitoneal administration of the drugs. Immediately after injection the animals were returned to the same cages, one mouse to a cage for isolation and 10 mice to a cage for aggregation. To maintain group size during the experiment, any dead mouse was replaced by another living animal.

Data summarized in Fig. 1 show that aggregation enhanced the acute lethality of imipramine and desipramine. Imipramine was less toxic than desipramine. Table 1 shows that the onset of clonic convulsions or death after a large dose of desipramine was significantly sooner after aggregation.

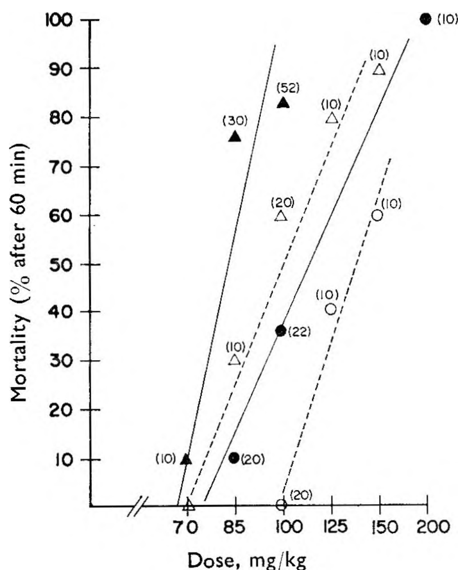

FIG. 1. Toxicity of imipramine and desipramine (doses as mg/kg) in aggregated and isolated mice. ——▲—— Desipramine aggregated. ——●——Desipramine isolated. - - -△- - - Imipramine aggregated. - - -○- - - Imipramine isolated.

TABLE 1. ENHANCEMENT OF DESIPRAMINE*-INDUCED CONVULSIONS AND LETHALITY BY AGGREGATION

	Mean latency (min) ± s.e.		
	Isolated	Aggregated	P
Convulsions	5·4 ± 0·37	3·5 ± 0·37	<0·01
Death	8·0 ± 0·50	5·1 ± 0·34	<0·01

* 200 mg/kg injected intraperitoneally into 10 mice in each group produced convulsions and death in all of the animals. Time of first clonic convulsion is given.

Imipramine and desipramine were more toxic to aggregated mice than to isolated mice. Previous experiments with amphetamine (Cohen & Lal, 1964; Lal, Ginnochio & Shefner, 1963; Mennear & Rudzik, 1966) and cocaine (Lal & Chessick, 1965) suggested that the toxicity of these compounds was related to the tissue catecholamines. Recently, dependence of desipramine toxicity on tissue catecholamines was reported (Lal & Brown, 1968). Thus, amphetamine, cocaine, and desipramine are not lethal in the animals depleted of catecholamines. It is speculated that the aggregation provides excessive sensory stimulation which causes release of central and peripheral catecholamines. Inactivation of these physiologically active amines by "reuptake" mechanisms (Kopin, 1964) is prevented by amphetamine (Axelrod & Tomchick, 1960; Carlsson, Dahlstrom & others, 1965; Glowinski, Iversen & Axelrod, 1966), cocaine (Macmillan, 1959; Whitby, Hertting & Axelrod, 1960), and imipramine-like drugs (Iversen, 1965). This can be expected to enhance and prolong the potent actions of the released catecholamines on target tissues, thereby increasing susceptibility of the animals to the toxicity of certain drugs. Enhancement of desipramine toxicity by noradrenaline has recently been reported by Jori (1966).

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The binding of imipramine to the outer membrane of blood platelets

SIR,—Imipramine has long been known to inhibit the uptake of noradrenaline into sympathetic nerve endings and 5-hydroxytryptamine (5-HT) into platelets (Marshall, Stirling & others, 1960; Stacey, 1961). It has been assumed that this action is brought about by inhibition of active transport at the outer cell membrane (Carlsson, 1966), however, direct evidence is lacking. We now present experimental evidence that [^{14}C]imipramine is rapidly accumulated at the outer membrane of the human blood platelet.

In two experiments platelets were incubated in 2×10^{-6} or 2×10^{-5}M [^{14}C]imipramine HCl (specific activity 25·4 μc/mg; Radiochemical Centre, Amersham) or 2×10^{-5}M [^{14}C]inulin (specific activity 3·08 μc/mg; New England Nuclear Corporation, Boston) for 1–60 min. The radioactivity in platelets and plasma was estimated by liquid scintillation spectrometry.

The inulin experiments determined the amount of drug which was trapped in the space between the platelets during separation from plasma by centrifugation, and a value of 0·57 μl/μl packed platelets was obtained after 10 or 60 min incubation. This figure is at the upper end of the range found by Born & Gillson (1959).

When comparable experiments were made with imipramine the "apparent" extracellular space was almost exactly 10 times greater (5·80 μl/μl packed platelets). As inulin is commonly used to estimate extracellular space, we propose that the difference between the values obtained with imipramine and inulin represents the binding of the former to the platelets. Therefore when calculating the extent of imipramine binding to platelets we have estimated the percentage of substance localized in or on the cells by subtraction of the proportion of drug trapped between the platelets as predicted from the inulin determinations. The results (Table 1), show that imipramine binds to platelets extremely rapidly with no further increase in accumulation upon prolonged incubation. As the time-course of binding reaches equilibrium within 1 min it is unlikely that imipramine enters the cells. This view is supported by results obtained when four samples of platelet-rich plasma were incubated with 2×10^{-6}M imipramine for 10 and 60 min and the labelled platelets subsequently washed with drug-free plasma. We found that the amount of imipramine retained was reduced from 8 to 1%.

TABLE 1. DISTRIBUTION OF IMIPRAMINE AND INULIN IN PLATELETS

Time of incubation (min)	Imipramine recovered (%)	Inulin recovered (%)	Imipramine bound (%)
1	8·22	0·78	7·44
10	7·36	0·58	6·78
60	8·86	0·55	8·31

Percentage figures refer to the proportion of radioactivity recovered in the platelets separated from 1 ml of platelet-rich-plasma by centrifugation at 2,000 × *g* for 5 min.
Each result is the mean of 2 values obtained in separate experiments.

Thus, the uptake of imipramine by platelets cannot be explained solely on the basis that the drug is confined to the interstices between the packed cells. The fact that the accumulation is almost instantaneous and that even after incubation for 1 hr, imipramine is easily displaced by washing, supports the belief that imipramine interferes with platelet and sympathetic function by an action on the outer membrane.

Mills & Roberts (1967) have clearly shown that imipramine is a potent inhibitor of platelet aggregation when studied under conditions similar to

those described here. It is probable that their results can be explained on the basis that imipramine, in binding to the cell membrane, prevents the access to the interior of the cell of compounds which cause aggregation, as well as inhibiting the release of ADP from the platelets as they suggested.

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β-Blocking agents on the pupil of the frog

SIR,—It is believed that the sympathetic effect on the dilator pupillae is mediated via α-receptors (Beaver & Riker, 1962; Ahlquist, 1966). The intravenous administration of α-blocking agents like phenoxybenzamine to dogs or rabbits, followed by an intraoccular injection of (−)-adrenaline, (−)-noradrenaline and (−)-isoprenaline inhibits mydriasis. Pretreatment with a β-blocking agent like dichloroisoprenaline in a similar way does not inhibit mydriasis (Bennett, Reinke & others, 1961). When the effect of applied catecholamines is observed on the isolated eye of the frog prepared according to Ehrmann (1905), evidence of the existence for β-receptors is uncovered.

One eye is placed in an isotonic saline solution and serves as a control. The other is placed in isotonic saline solution in which the drug has been dissolved. Propanolol in a concentration of 2 to 5 $\times 10^{-5}$ provokes miosis within 3 hr which 8–12 hr later develops into a complete closure of the pupil; while isoprenaline provokes mydriasis in a concentration of 0·5 to 1 $\times 10^{-5}$. This latter action is partially inhibited in eyes which have been placed in the propranolol solution.

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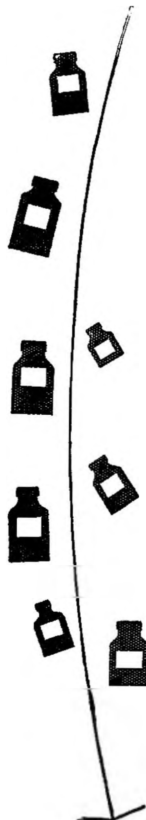

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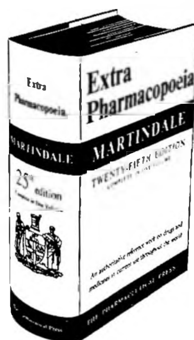

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