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Central hypotensive effect of α-methyldopa

M. HENNING AND P. A. VAN ZWIETEN

In cats under chloralose anaesthesia L-α-methyldopa (20 mg/kg) was infused for 1 hr into the left vertebral artery. One to 3 hr after the end of the infusion a gradual and significant lowering of mean arterial blood pressure was observed. The dopamine and noradrenaline contents of the brain were significantly reduced while brain 5-hydroxytryptamine and heart noradrenaline concentrations remained normal. The same low dose of L-α-methyldopa infused into a systemic vein did not affect the blood pressure. However, brain dopamine and noradrenaline were depleted to the same extent as observed after infusion into the vertebral artery. Intravenous infusion of a large dose of L-α-methyldopa (200 mg/kg) did not significantly alter mean arterial blood pressure but lowered brain dopamine, noradrenaline and 5-hydroxytryptamine levels. No effect on heart noradrenaline was observed. Infusion of saline or the D-isomer of α-methyldopa (20 mg/kg) into the vertebral artery had no effect on blood pressure or tissue monoamines.

THE mechanism underlying the antihypertensive action of L-α-methyl-3,4-dihydroxyphenylalanine (α-methyldopa) in animals and in man has been subject to much research in recent years but still presents a number of unexplained features. The subject has been extensively reviewed by Sourkes (1965), Muscholl (1966), Holtz & Palm (1966) and Stone & Porter (1966; 1967).

It is commonly agreed that the hypotensive effect of α-methyldopa is mediated through interference with the sympathetic system in general. However, the exact site of action has not been established. Opinions differ as to the function of the peripheral sympathetic nerves after treatment with α-methyldopa, but there seems to be only a moderate impairment of peripheral adrenergic mechanisms (see reviews mentioned above; cf. also Haefely, Thoenen & Hürlimann, 1967; Haefely, Hürliman & Thoenen, 1967). On the other hand, several obvious effects of α-methyldopa on the central nervous system have been reported.

In vivo, the metabolism of catecholamines is affected by α-methyldopa in a number of different ways. Tissue levels of dopamine, noradrenaline and 5-hydroxytryptamine (5-HT) are decreased upon administration of the drug to animals (Smith, 1960; Porter, Totaro & Leiby, 1961; Hess, Ozaki & Udenfriend, 1960; Hess, Connamacher & others, 1961). The depletion of dopamine and noradrenaline occurs mainly through a stoichiometric exchange of these amines with the metabolites of α-methyldopa, i.e., α-methyldopamine and α-methylnoradrenaline, respectively (Carlsson & Lindqvist, 1962; Carlsson, 1964), whereas the lowering of 5-HT may be the result of inhibition of synthesis (Sharman & Smith, 1962; Roos & Werdinius, 1963; Burkard, Gey & Pletscher, 1964).

The interrelation of these biochemical changes and the functional effects of α-methyldopa is also subject to debate. Apparently, the drug must undergo decarboxylation to form α-methyldopamine and α-methylnoradrenaline or both, in order to exert its hypotensive and catecholamine depleting effects (Davis, Drain & others, 1963; Henning, unpublished

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experiments). Carlsson & Lindqvist (1962) suggested that the amines formed on metabolism of α-methyldopa may take over the function of dopamine and noradrenaline in the brain and later Day & Rand (1963) extended this assumption to the peripheral nerves as well. It has also been shown that in these nerves, α-methylnoradrenaline formed from α-methyldopa is released after electrical stimulation (Muscholl & Maître, 1963) and a number of investigators have stated that the activity of α-methylnoradrenaline on the adrenergic receptors is less than that of noradrenaline (Mueller & Horwitz, 1962; Day & Rand, 1964; Brunner, Hedwall & others, 1966; 1967). This release of a substitute or "false" transmitter of inferior quality has been proposed as an explanation for the hypotensive effect of α-methyldopa (Day & Rand, 1963; review by Muscholl, 1966). However, the time relation between the noradrenaline depletion and the decrease in blood pressure is poor, the latter effect being shorter-lasting than the former (Torchiana, Porter & others, 1965; Henning, 1967). This discrepancy implies that the false transmitter concept in its simplest outline is not sufficient to explain the antihypertensive action of α-methyldopa.

Considering the uncertainty about the site of action of the blood pressure lowering property of α-methyldopa, it was thought of interest to investigate whether a possible central nervous component significantly contributes to this action. In previous studies the administration of drugs into a vertebral artery in the cat has been used to investigate whether hypotensive effects of vasoactive drugs are mediated by the central nervous system (Zwieten, Bernheimer & Hornykiewicz, 1966; Reis & Zwieten, 1967; Sattler & Zwieten, 1967). In the present work a low dose of α-methyldopa has been infused into a vertebral artery in chloralose anaesthetized cats. The effects of this procedure on arterial blood pressure and tissue monoamines have been compared with those obtained after infusion of the same low dose of α-methyldopa into a systemic vein. Control experiments have been made with infusions of saline or the D-isomer of α-methyldopa. A significant lowering of blood pressure was seen only after administration of L-α-methyldopa into a vertebral artery although brain catecholamines were decreased both in this case and upon intravenous infusion. Part of the results have been reported in preliminary communications previously (Henning & Zwieten, 1967a,b).

Experimental

MATERIAL AND METHODS

The experiments were made on cats of either sex (2·0–4·5 kg) anaesthetized with chloralose (60–70 mg/kg i.p.). Artificial respiration was applied via a tracheal cannula. The blood pressure in the left femoral artery was recorded continuously by a Statham pressure transducer type P23Dc and a Grass Polygraph.

For infusion of solutions into the left vertebral artery, a polyethylene catheter was introduced into the left subclavian artery, which was exposed

after thoracotomy. All its side-branches were ligated with the exception of the vertebral artery. Solutions infused slowly into the catheter may be expected to flow into the vertebral artery since the blood flow in the subclavian artery prevents the infused solution entering the heart and thus into the peripheral circulation. Accordingly, the drugs reach the brain stem in high concentrations. The exact position of the catheter is shown in Fig. 1. Details of the surgical procedure have been described

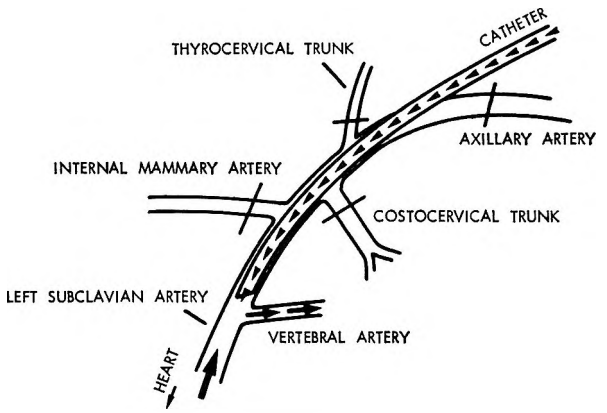

FIG. 1. Schematic representation of the technique used for infusion of drugs into the left vertebral artery. The left subclavian artery is exposed after thoracotomy. The axillary artery and all its side-branches except the vertebral artery are ligated. A polyethylene catheter is introduced into the subclavian artery so that the tip lies at the level of the vertebral artery.

previously (Zwieten & others, 1966; Reis & Zwieten, 1967; Henning & Zwieten, 1967a; Sattler & Zwieten, 1967). Drugs dissolved in saline (5–7 ml) were infused into the catheter over a period of about 1 hr. In a preliminary experiment (not included here) rapid injection of α-methyldopa into the catheter had no effect on blood pressure. For intravenous infusion we used the left femoral vein.

The animals were killed by cutting the great vessels to the heart about 3 hr after the end of the infusion. The amine contents of the brain (cerebellum removed) and of the right ventricle of the heart were measured. In some experiments the brain was sectioned immediately behind the mamillary bodies from the ventral surface and in front of the anterior corpora quadrigemina from the dorsal surface. The two parts of the brain, "forebrain" and "lower brain stem," were then analysed separately. Noradrenaline (Bertler, Carlsson & Rosengren, 1958), dopamine (Carlsson & Lindqvist, 1962), and 5-HT (Andén & Magnusson, 1967) were measured. α-Methyldopamine was determined by utilizing the observation (Carlsson & Lindqvist, 1962) that this amine behaves essentially like dopamine on the cation exchange resin columns used for separation of noradrenaline and dopamine, whereas it behaves like noradrenaline in the fluorimetric assay and can be distinguished in this way from dopamine.

Results

Blood pressure. Changes in blood pressure observed upon the various treatments are represented in Fig. 2. Infusion of L-α-methyldopa 20 mg/kg into the left vertebral artery produced a decrease in mean arterial blood pressure which was slow in onset; usually no change in pressure was observed during the 1 hr infusion. Within the first hr after the end of the infusion a gradual fall in blood pressure occurred and 1 hr after the infusion had been terminated the average decrease in blood pressure was 17 mm Hg (s.e. 3·3, n = 10). During the following hours a further diminution in pressure was seen, the mean decrease 2 and 3 hr after the end of the infusion amounting to 27 mm Hg (s.e. 5·9, n = 10) and 36 mm Hg (s.e. 6·0, n = 10), respectively. Calculated in per cent of the pre-infusion blood pressure levels, which were about the same in the five series of experiments (Fig. 2), these decreases amounted to 12% (s.e.

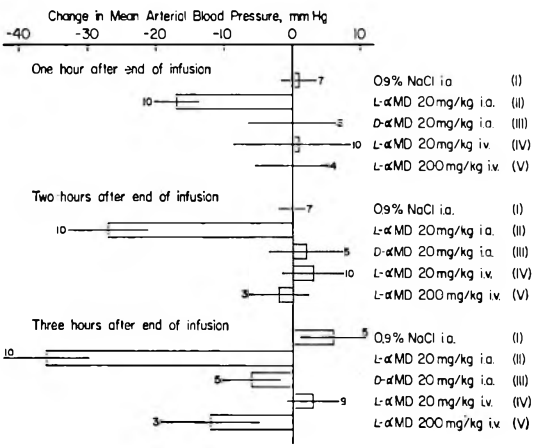

FIG. 2. Changes in mean arterial blood pressure after infusion of various drugs into the left vertebral artery (i.a.) or into the femoral vein (i.v.) of anaesthetized cats. The values are means with s.e.; number of experiments is indicated with the small figures.

Analysis of variance of the changes at each interval after the end of the infusion shows: After 1 hr (II) differs significantly from (I) and (IV) ($P < 0{\cdot}1\%$) and from (III) and (V) ($P < 1\%$). After 2 and 3 hr (II) differs significantly from (I), (III) and (V) ($P < 0{\cdot}1\%$) and from (V) ($P < 2{\cdot}5\%$). (I), (III), (IV) and (V) were not significantly different from each other at any interval ($P > 10\%$).

Initial blood pressure levels: (I) 135 mm Hg (s.e. 11·7), (II) 139 mm Hg (s.e. 5·5), (III) 137 mm Hg (s.e. 11·0), (IV) 150 mm Hg (s.e. 3·0), (V) 130 mm Hg (s.e. 5·8).

2·4), 19% (s.e. 3·8) and 27% (s.e. 4·1), respectively. In a few experiments the blood pressure was followed for as long as 4–5 hr after the end of the infusion and in these experiments no further decrease was observed.

The lowering of blood pressure after infusion of L-α-methyldopa in this dose was at all intervals statistically significant (analysis of variance; P values are given in Fig. 2) from the changes in the control experiments (infusion of saline or D-α-methyldopa) and after systemic infusion.

In another series of experiments L-α-methyldopa (20 mg/kg) was infused into the femoral vein. In this series the vertebral artery was also cannulated (sham operation) but no infusion was given. In control experiments D-α-methyldopa (20 mg/kg) or saline were infused into the left vertebral artery. These procedures provoked only small and variable changes in blood pressure which were not statistically different.

Intravenous infusion of a large dose of L-α-methyldopa (200 mg/kg) slightly lowered the blood pressure after 3 hr (mean decrease 12 mm Hg, s.e. 7·3, n = 4) but the change did not differ statistically from the control experiments.

Tissue monoamines. The levels of heart noradrenaline, whole brain noradrenaline, dopamine and 5-HT after the various types of treatment are given in Table 1. Control values were obtained from the animals infused with saline.

TABLE 1. LEVELS OF TOTAL BRAIN NORADRENALINE (NA), DOPAMINE (DA), 5-HYDROXYTRYPTAMINE (5-HT) AND α-METHYLDOPAMINE (α-M-DA) AND OF HEART NA AFTER VARIOUS TREATMENTS AS INDICATED. The values are means ± s.e. and number of experiments. P values were calculated by analysis of variance.

Treatment	Total brain				Heart (right ventricle)
	NA	DA	α-M-DA	5-HT	NA
A. Controls (0·9% NaCl in vertebral artery)	0·23 0·014 (10)	0·37 0·033 (9)	0·03 0·02 (3)	0·29 0·017 (10)	1·19 0·138 (9)
B. L-α-Methyldopa 20 mg/kg in vertebral artery	0·16 0·010 (12)	0·27 0·017 (12)	0·28 0·040 (6)	0·27 0·026 (10)	1·48 0·137 (12)
C. D-α-Methyldopa 20 mg/kg in vertebral artery	0·23 0·011 (6)	0·28 0·021 (5)	—	0·26 0·026 (5)	1·82 0·406 (5)
D. L-α-Methyldopa 20 mg/kg in femoral vein	0·17 0·013 (10)	0·27 0·024 (9)	0·27 0·022 (4)	0·26 0·018 (10)	1·34 0·117 (10)
E. L-α-Methyldopa 200 mg/kg in femoral vein	0·13 0·008 (4)	0·15 0·013 (4)	0·65 0·078 (4)	0·12 0·018 (4)	0·86 0·051 (4)
	P %	P %	P %	P %	
A–B	<0·1	<0·5	<0·1	>10	n.s.
A–C	>10	<5	—	>10	
A–D	<0·1	<1	<1	>10	
A–E	<0·1	<0·1	<0·1	<0·1	
B–C	<0·1	>10	—	>10	
B–D	>10	>10	>10	>10	
B–E	>10	<0·1	<0·1	<0·1	
C–D	<0·5	>10	—	>10	
C–E	<0·1	<0·1	—	<1	
D–E	<10	<0·1	<0·1	<0·5	

Three hr after an infusion of L-α-methyldopa (20 mg/kg) into a vertebral artery there was a significant, though moderate reduction of brain dopamine and noradrenaline while brain 5-HT and heart noradrenaline remained normal (for P values, see Table 1). Similarly, the intravenous infusion of the same dose of L-α-methyldopa also lowered brain dopamine and noradrenaline significantly, but brain 5-HT and heart noradrenaline

remained unaffected (for P values, see Table 1). There was no difference between the degree of amine depletion in these experiments and in those in which L-α-methyldopa was infused intra-arterially. When D-α-methyldopa was administered into the vertebral artery no significant changes were observed in the brain or heart amine contents. The intravenous infusion of a large dose of L-α-methyldopa (200 mg/kg) gave a significant reduction of all the amines studied in the brain (for P values, see Table 1). The noradrenaline content in the heart also seemed lower in this series than in the other four, but the difference was not significant.

When it was found that infusion of L-α-methyldopa into the vertebral artery and intravenous infusion of the same dose produced similar degrees of amine depletion, the lower brain stem and forebrain were analysed separately in some experiments. The results are summarized in Table 2.

TABLE 2. LEVELS OF NORADRENALINE (NA), DOPAMINE (DA) AND 5-HYDROXYTRYPTAMINE (5-HT) IN LOWER BRAIN STEM AND FOREBRAIN AFTER TREATMENTS AS INDICATED.

Treatment	Lower brain stem			Forebrain		
	NA	DA	5-HT	NA	DA	5-HT
Control (0·9% NaCl in vertebral artery) ..	0·26 0·032 (6)	0·08 0·005 (5)	0·64 0·066 (6)	0·24 0·016 (7)	0·44 0·016 (6)	0·27 0·017 (7)
L-α-Methyldopa 20 mg/kg in vertebral artery ..	0·21 0·012 (6)	0·09 0·023 (6)	0·66 0·118 (5)	0·18 0·010 (6)	0·31 0·025 (6)	0·27 0·031 (6)
L-α-Methyldopa 20 mg/kg in femoral vein ..	0·23 0·033 (6)	0·07 0·005 (5)	0·58 0·067 (6)	0·20 0·010 (6)	0·32 0·025 (6)	0·25 0·012 (6)

No difference between the effect of intra-arterial or intravenous administration of α-methyldopa was observed.

In a few experiments the level of α-methyldopamine in the brain was also measured (Table 1). Intravenous and intra-arterial administration of L-α-methyldopa 20 mg/kg gave rise to similar concentrations of α-methyldopamine. After intravenous infusion of 200 mg/kg L-α-methyldopa the α-methyldopamine concentration in the brain was significantly higher than after the smaller dose (Table 1).

Discussion

These results show that a small dose of L-α-methyldopa when infused into the vertebral artery lowers the mean arterial blood pressure of cats anaesthetized with chloralose. In a preliminary experiment it was found that a rapid injection of the same dose of α-methyldopa had no effect. Since infusion of saline or D-α-methyldopa into the vertebral artery had no influence on the blood pressure in identical experiments, it seemed that the effect of L-α-methyldopa was specific; it is generally agreed that the biological activity of α-methyldopa resides in the L-isomer (Porter & others, 1961; Sjoerdsma, 1961; Gillespie, Oates & others, 1962; Sjoerdsma, Vendsalu & Engelman, 1963). Furthermore, in our experiments the lowering of blood pressure after L-α-methyldopa was slow in

onset. The effect had probably reached its maximum about 3 hr after the infusion had been terminated, since no additional decrease occurred in the few experiments in which the blood pressure was followed for a further 1–2 hr. A similar time course is observed when large single doses of α-methyldopa are administered to conscious animals (Goldberg, DaCosta & Ozaki, 1961; Kroneberg, 1963; Davis & others, 1963; Torchiana & others, 1965; Henning, 1967) or to man (Schaub, Nager & others, 1962; Cannon, Whitlock & others, 1962; Onesti, Brest & others, 1964). The relative magnitude of the hypotensive response after infusion of L-α-methyldopa into the vertebral artery was about the same as that observed after systemic administration of much larger doses in conscious animals. In our experiments, the intravenous infusion of a relatively large dose of the drug failed to lower the blood pressure significantly. This observation is in agreement with most previous investigations. However, Miele (1966) observed a significant decrease of blood pressure in cats under urethane anaesthesia after oral administration of α-methyldopa.

In the present investigation, α-methyldopa significantly lowered whole brain dopamine and noradrenaline either after infusion into the vertebral artery, or after intravenous infusion, and the depletion was of the same moderate degree. Moreover, the levels of α-methyldopamine were about the same in these two types of experiments. When the lower brain stem was analysed separately there was still no significant difference in amine content after the two routes of administration. These findings are puzzling in view of the clear-cut effect on blood pressure after infusion into the vertebral artery. However, in view of the small number of animals investigated, the evidence is not conclusive. Possibly, a more extensive and detailed analysis of various parts of the brain stem might have disclosed a correlation between the lowering of blood pressure and the decrease in amine content. It seems reasonable to conclude, however, that there exists in the region supplied by the vertebral artery a structure which is influenced by infusion of α-methyldopa into this vessel in such a way as to bring about a reduction in blood pressure. The determination of the nature and exact localization of this effect requires further investigation. This component of the hypotensive effect of α-methyldopa is not necessarily related to the gross action of the drug on brain monoamines. A potentiation of anaesthesia by α-methyldopa seems rather unlikely since the intravenous infusion of 200 mg/kg does not affect blood pressure.

There is ample evidence for an effect of α-methyldopa on the central nervous system although the relative importance of this component for the hypotensive action has not been established. Thus, in animal experiments, the drug potentiates barbiturate narcosis and causes sedation and suppression of learned behaviour (Sourkes, 1965; Hanson & Henning, 1967). The α-methylated amine metabolites of α-methyldopa, α-methyldopamine and α-methylnoradrenaline, deplete the noradrenaline from sympathetically innervated organs but not from the brain; they have no antihypertensive effect in rats (Brunner & others, 1966; 1967; Henning,

1967). In man, α-methyldopa inhibits various haemodynamic reflexes involving both central and peripheral nervous mechanisms (Dollery & Harington, 1962; Sannerstedt, Varnauskas & Werkö, 1962; Mason & Braunwald, 1964; Shapiro & Krifcher, 1964). Clinical experience with the drug has also revealed a number of predominantly central nervous effects. These include sedation, psychiatric symptoms, Parkinsonism and abnormal lactation (Sourkes, 1965; Horwitz, Pettinger & others, 1967).

After infusion of the D-isomer of α-methyldopa the amine levels in the brain were the same as those observed after saline infusion. D-α-Methyldopa neither affected the blood pressure nor the concentration of monoamines in other species, or in man (Porter & others, 1961; Sjoerdsma, 1961; Gillespie & others, 1962; Sjoerdsma & others, 1963). It is not known whether in the cat D-α-methyldopa penetrates the blood-brain barrier to the same extent as does the L-isomer.

None of the treatments affected the noradrenaline content of the heart significantly. Thus, it appears that the brain is more sensitive to the noradrenaline-depleting action of small doses of α-methyldopa than is the heart. For reserpine-induced depletion, the opposite seems to be true (Carlsson, Rosengren & others, 1957; Carlsson, 1965). Central monoamine neurons generally appear to operate with a higher metabolic rate than the peripheral adrenergic nerves. The present observations may thus be interpreted to show that α-methyldopa depends more than does reserpine on the rate of metabolism for its catecholamine depleting action.

The results described in the present paper suggest that the blood pressure lowering effect of α-methyldopa may be, at least in part, of central origin. The exact mechanism of this effect remains unknown. The centrally mediated effect does not necessarily exclude the contribution of impaired peripheral sympathetic function to the hypotensive action of α-methyldopa.

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Polymorphism of cephaloridine

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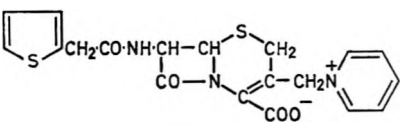
Infrared spectra of Nujol mulls and X-ray powder photographs have shown that cephaloridine can exist in at least six different crystalline forms. The preparation, interconversion and properties of these forms are discussed. Proton magnetic resonance spectroscopy has been used to check their chemical identity.

INFRARED measurements have shown that cephaloridine (I) (Martin & Shaw, 1965; Spencer, Siu & others, 1967), like other cephalosporin C derivatives, when recrystallized under different conditions gives rise to several crystalline forms (Green, Page & Staniforth, 1965); the most important of these we have called the α-, β-, δ-, ϵ-, ζ- and μ-forms.



I

The α- and β-forms are prepared by treating with triethylamine, solutions of cephaloridine hydronitrate in *NN*-dimethylacetamide and *NN*-dimethylformamide, respectively. The same treatment of solutions of cephaloridine hydronitrate in dimethyl sulphoxide, ethylene glycol, diethylene glycol and benzyl alcohol gives the respective solvates all having the ζ-form; while from methanolic solutions a methanolate, the μ-form, is obtained. Recrystallization of any of the forms from water leads to the δ-form, while prolonged drying of the μ-form *in vacuo* leads to the ϵ-form. The various forms may be inter-converted *via* the μ-form. Thus, treatment of the μ-form with *NN*-dimethylacetamide, *NN*-dimethylformamide, water vapour or dimethyl sulphoxide gives the α-, β-, δ- and ζ-forms, respectively; these may be reconverted to the μ-form by treatment with methanol or methanol vapour. Details for the preparation and interconversion of the six crystalline forms are given in the Experimental section.

SPECTROSCOPIC MEASUREMENTS

Dickson, Page & Rogers (1955) have shown that the various crystalline forms of a polymorphic substance give different infrared spectra when examined in the solid state as Nujol mulls or alkali halide discs, but identical spectra when examined in solution. However, since water transmits over only a narrow range in the infrared region, aqueous solutions of water-soluble compounds, such as cephaloridine, do not give solution spectra that are sufficiently reliable for checking chemical identity and, hence, confirming the chemical identity of the polymorphs.

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We have found that a combination of solid-state infrared with proton magnetic resonance (pmr) measurements on heavy-water solutions provides a better test for polymorphism in water-soluble compounds. Thus, the crystalline forms of cephaloridine may be distinguished by solid-state infrared (Fig. 1) or X-ray measurements (Table 1), and the chemical identity (but see below) of the different forms established by pmr measurements on heavy water solutions. The pmr measurements not only confirm the structure of cephaloridine (Green, Page & Staniforth, 1965), but also yield quantitative information on solvent and other impurities.

TABLE 1. X-RAY POWDER DATA FOR α-, β-, ϵ-, ζ- AND μ-FORMS OF CEPHALORIDINE (CU-Kα-RADIATION)

α-Form		α-Form		β-Form		β-Form		ε-Form		ζ-Form		μ-Form	
d (Å)	I*	d (Å)	I*	d (Å)	I	d (Å)	I	d (Å)	I	d (Å)	I	d (Å)	I
14·5	m	3·47	wd	14·5	m	3·58	vw	10·2	wd	7·4	vw	7·3	m
7·4	m	3·39	m	7·4	w	3·52	vw	7·7	md	6·9	w	6·7	w
6·4	w	3·35	w	6·8	w	diff. 3·50	w	6·3	wd	6·2	wd	5·8	vw
5·7	s	3·29	vw	6·01	w	3·42	w	5·4	s	5·8	vw	5·45	md
5·55	s	3·12	wd	5·75	s	3·41	w	5·25	w	5·6	w	5·15	md
5·25	s	2·96	vw	5·65	w	3·35	vw	5·1	vwd	5·4	vwd	4·5	md
4·9	w	2·82	wd	diff. 5·3	wd	3·30	vw	4·8	m	5·2	w	4·4	m
4·8	vw	2·76	vw	5·29	vw	3·22	vw	4·4	vw	4·95	w	4·0	vw
4·5	m	2·72	vw	4·95	s	3·18	vw	4·25	w	4·7	vw	3·90	m
4·2	m	2·67	vw	4·85	m	3·02	w	4·15	w	4·5	w	3·65	w
4·05	m	2·64	w	4·5	w	2·86	w	4·0	w	4·4	w	3·60	vw
3·87	w	2·57	vw	4·3	s	2·82	w	3·90	w	4·05	w	3·50	w
3·83	vw	2·54	w	4·25	m	2·78	w	3·65	w	3·95	w	3·35	m
3·74	w	2·51	vvw	4·1	m	2·77	vw	3·60	w	3·80	w	3·25	m
3·70	w	2·47	vwd	3·96	m	2·68	w	3·55	vwd	3·75	w		
3·67	wd	2·43	w	3·85	m	2·58	w	3·47	vw	3·55	m		
3·55	vw			3·79	wd	2·56	w	3·40	w				
				3·72	wd	2·52	vwd	3·30	w				
								3·13	m				
								2·82	wd				

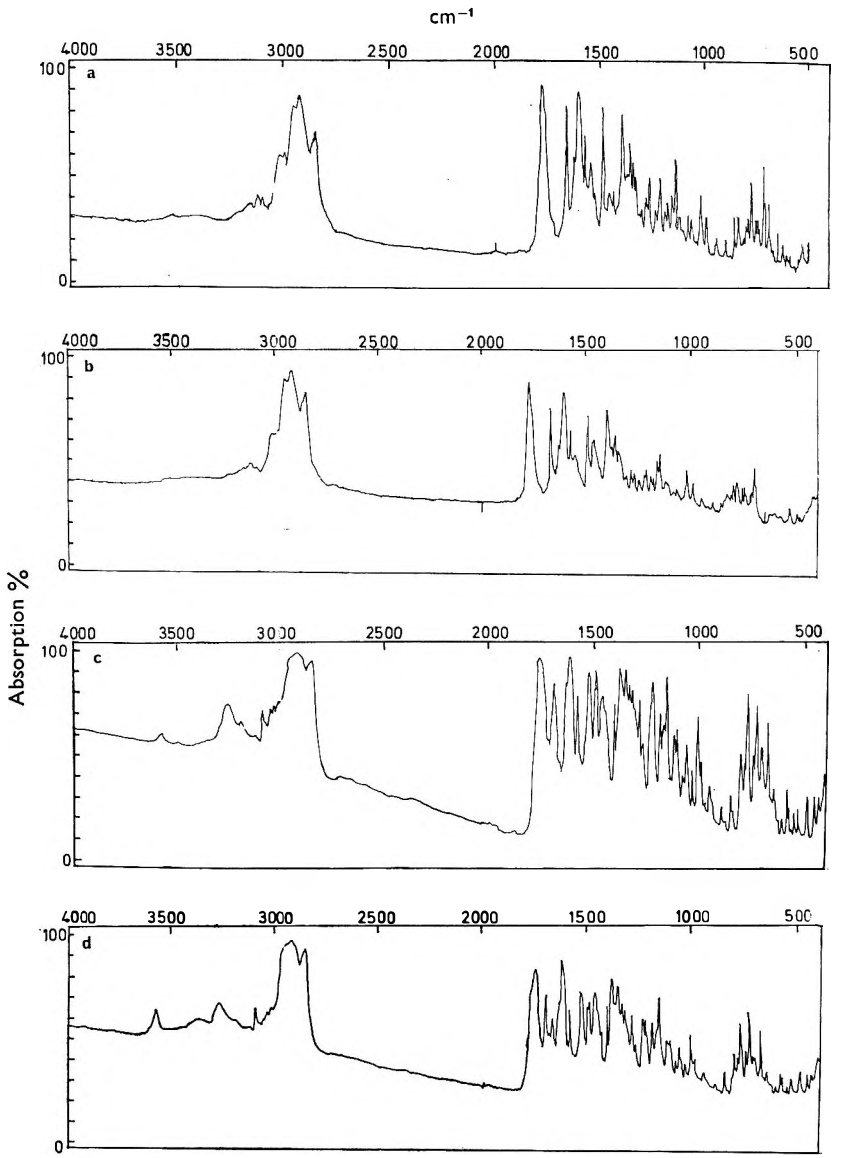
* s = strong, m = medium, w = weak, vw = very weak, d = diffuse line.

Proton magnetic resonance spectroscopy showed that although the μ-form of cephaloridine contains about one mole of methanol and the ζ-form about one mole of either dimethyl sulphoxide, ethylene glycol, or diethylene glycol, depending on the solvent used for its preparation, the α-, β-, anhydrous δ- and ϵ- forms contain less than 0·1 mole (i.e. non-stoichiometric amounts) of solvent. The α-form is characterized by containing about 0·05 mole of *NN*-dimethylacetamide. This small amount of "impurity," which cannot be removed by prolonged drying at 10^{-5}–10^{-6} mm, is apparently able to "lock" the cephaloridine molecule in a particular crystal lattice. However, if the cephaloridine is reprecipitated or recrystallized from some other solvent, the cephaloridine takes on the crystal form associated with the new solvent.

Mesley (1965) has reported somewhat similar non-stoichiometric chloroform-adducts of hydrocortisone and dexamethasone, which are believed on thermogravimetric evidence to be clathrates. Chapman (1965) mentions that polymorphism in lipids often depends on the presence

or absence of impurities, but unfortunately does not take the matter further.*

The infrared spectra of Nujol mulls of the six crystalline forms of cephaloridine differ considerably (see Fig. 1). The α- and β-forms show



* After this paper was submitted for publication two papers by M. Kuhnert-Brandstätter and H. Grimm (*Mikrochim. Acta*, 1968, 115–126; 127–139) appeared in which solvent-containing crystal forms, "pseudopolymorphic" forms, of various steroids are described.

FIG. 1.—*continued.*

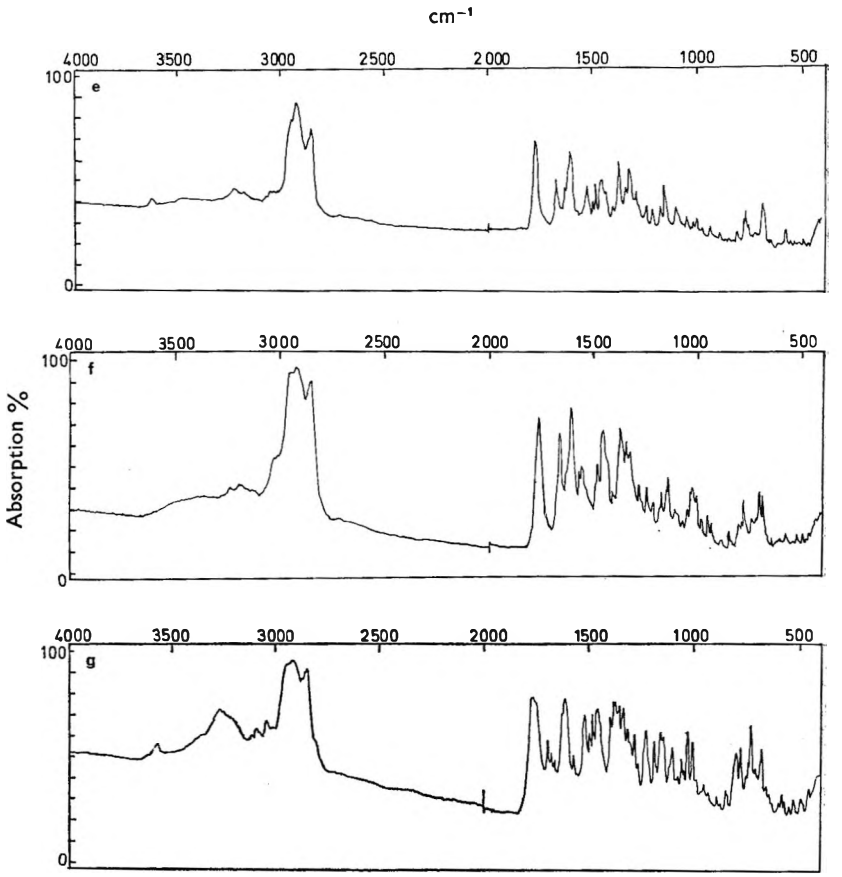


FIG. 1. Infrared spectra of Nujol mulls of (a) α-, (b) β-, (c) anhydrous δ-, (d) equilibrated δ- ($\frac{3}{4}$ mole of water), (e) ϵ-, (f) ζ- (*ex* dimethyl sulphoxide) and (g) μ-forms of cephaloridine.

a broad band for bonded N–H at about 3,120 cm^{-1} and no O–H absorption, confirming that the two forms are anhydrous. They give intense β-lactam (1,774–1,776 cm^{-1}), ionized carboxyl (1,602 cm^{-1}) and secondary amide (1,666–1,670 and 1,548–1,554 cm^{-1}) bands and may be distinguished by strong C–H bending bands at 718 and 700 cm^{-1}, respectively.

The spectrum of the μ-form, which contains about 1 mole of methanol, is characterized by broad O–H and N–H absorption bands at 3,350 and 3,270 cm^{-1}, secondary amide peaks at 1,690 and 1,518 cm^{-1} and C–H bending bands at 730 and 680 cm^{-1}. The O–H and C–O bands at 3,270 and 1,028 cm^{-1}, respectively, which disappear when the methanol is removed and the sample converted to the ϵ-form, are attributed to methanol. The ϵ-form shows a weak N–H peak at 3,225 cm^{-1} and secondary amide bands at 1,678 and 1,528 cm^{-1}; the C–H bending peak is at 690 cm^{-1}. In all forms, the carbonyl bands for the β-lactam and

ionized carboxyl groups are much stronger than the corresponding secondary amide bands.

The anhydrous δ-form (from water) and μ-form (from methanol) are markedly hygroscopic; the weak peaks at about 3,575 cm^{-2} in Fig. 1c and g are attributed to traces of water picked up during the mulling operation. Samples of the δ-form in equilibrium with the normal laboratory atmosphere are repeatedly found to contain about $\frac{3}{4}$ mole of water, and some effort has been made to discover whether a discrete hydrate exists; so far without decisive result.

Measurements of partial pressures of water vapour in equilibrium with solids of various water contents give no indication of the formation of stoichiometric hydrates by the α-, β- or δ-forms. Thermogravimetric analysis of the hydrated δ-form gives some support to the existence of a hydrate containing $\frac{3}{4}$ mole of water; but the principal positive evidence for such a hydrate comes from infrared spectra and from the demonstration of a distinct break at about $\frac{3}{4}$ mole in the rate of water-uptake plot, measured on δ-form in contact with a saturated atmosphere. The evidence from powder and single crystal X-ray data is, however, rather strongly against this hydrate having a definite stoichiometry.

The infrared spectrum of an anhydrous sample of the δ-form shows N–H bands at 3,260 and 3,196 cm^{-1} and no other absorption between 3,600 and 3,100 cm^{-1}. The relatively strong 3,260 cm^{-1} band probably accords with the stretching vibration of an N–H group that is *trans* N–H . . . O=C hydrogen-bonded, but the weaker 3,196 cm^{-1} band represents either the stretching vibration of an N–H group that is *cis* N–H . . . O=C hydrogen-bonded (Darmon & Sutherland, 1949) or the Fermi resonance of the secondary amide I and II vibrations (Cannon, 1960). Absorption bands for the β-lactam and ionized carboxyl groups appear at 1,762 and 1,620 cm^{-1}, respectively, and for the secondary amide I and II vibrations at 1,695 and 1,525 cm^{-1}, respectively.

On hydration with up to about $\frac{3}{4}$ mole of water, characteristic O–H stretching and O–H bending bands for water-of-crystallization appear at 3,575 and 3,366, and at 1,665 cm^{-1}, respectively; the intensity of these bands is directly related to the water-content of the sample as measured by the Karl Fischer method. The water-of-crystallization hydrogen-bonds onto the carbonyl of the β-lactam ring and causes a decrease in the intensity of the carbonyl band at 1,762 cm^{-1} and a corresponding increase in the intensity of a new carbonyl band at 1,745 cm^{-1}. The relative intensity of the two carbonyl bands provides a measure of the amount of free and hydrogen-bonded β-lactam carbonyl. In the hydrate containing $\frac{3}{4}$ mole of water, the absorption band for unbonded β-lactam carbonyl appears as a shoulder on, and has about one-third of the intensity of, that for the bonded β-lactam carbonyl. The secondary amide and ionized carboxyl bands are unaffected by water uptake.

If the water-content of the δ-form is further increased up to 4 moles, the general pattern of the water bands at 3,575 and 3,366 cm^{-1} and of the β-lactam bands at 1,762 and 1,745 cm^{-1} does not change significantly. The additional water, which gives rise to broad absorption centred at

3,380 and 1,665 cm^{-1} and does not hydrogen-bond to the β-lactam carbonyl, is held less strongly than the first $\frac{3}{4}$ mole and is readily lost when the sample is exposed to an atmosphere of normal humidity, the spectrum reverting to that of the equilibrium form containing about $\frac{3}{4}$ mole of water.

Similar results were obtained with a specimen of cephaloridine in which the imido hydrogen had been replaced by deuterium. The anhydrous material showed N–D bands at 2,444 and 2,400 cm^{-1} and displaced amide I and II bands at 1,690 and 1,418 cm^{-1}, respectively; the other bands above 1,500 cm^{-1} had essentially the same frequencies as those for the δ-form of undeuterated cephaloridine. The relatively strong 2,400 cm^{-1} band is probably associated with the stretching vibration of an N–D group that is *trans* N–D . . . O=C hydrogen bonded, but the weaker 2,444 cm^{-1} band may be associated with Fermi resonance of the deuterated amide II′ and amide III′ vibrations. When deuterated cephaloridine is equilibrated with ordinary water, the water again hydrogen-bonds onto, and displaces from 1,762 to 1,745 cm^{-1} the absorption band for, the β-lactam carbonyl. The relative intensities of the β-lactam bands suggest that the sample has about $\frac{3}{4}$ mole of water. The water-of-crystallization bands that appear at 3,575, 3,368 and 1,664 cm^{-1} are similar to those shown by ordinary hydrated cephaloridine.

If deuterated cephaloridine is equilibrated with heavy water, the β-lactam carbonyl band splits as for the ordinary hydrate, but the O–H bands are replaced by O–D bands at 2,645, 2,630, 2,478 and 1,490 cm^{-1}. The δ-form of ordinary cephaloridine forms a similar "hydrate" with heavy water.

These results suggest that in every group of four molecules in the δ-form of cephaloridine, the β-lactam carbonyl groups of three can form hydrogen bonds with water and thereby prevent the fourth from doing so.

X-RAY MEASUREMENTS ON THE δ-FORM

X-ray powder photographs were taken of anhydrous, equilibrated ($\frac{3}{4}$ mole of water) and "wet" (about 1·0 mole of water) specimens of the δ-form suitably encapsulated, using a Guinier camera and Fe–Kα radiation (see Table 2).

Single-crystal studies were confined to the equilibrated form, but, as the crystals were small fragile flakes and always seemed to incorporate some strains, the spots on the film were not sharp. The thermal attenuation was also marked. Weissenberg photographs established that these crystals have Laue symmetry *mmm* and screw absences were noted on each axis to $h = 8$, $k = 5$, $l = 30$, so that the space group is uniquely identified as $P2_12_12_1$. The cell parameters were estimated as $a = 7{\cdot}2_9$, $b = 8{\cdot}1_9$ and $c = 32{\cdot}1$ Å to an accuracy of little better than 1%. By noting the relative strengths of the single-crystal reflexions it was possible to index the first 26 lines ($d \geqslant 3{\cdot}24$ Å) in its powder pattern. Of these, 22 lines were uniquely indexed and were used to calculate by least-squares the cell parameters and standard deviations in Table 3. With this as a guide it has been possible to index the powder patterns of the "wet" and

TABLE 2. OBSERVED AND CALCULATED POWDER DATA FOR "WET" (ABOUT 1 MOLE OF WATER), EQUILIBRATED ($\frac{3}{4}$ MOLE OF WATER) AND ANHYDROUS δ-FORMS OF CEPHALORIDINE (Fe-Kα-RADIATION)

	"Wet"				Equilibrated				Anhydrous			
hkl	d(Å)	I*	$10^5 \times \sin^2\theta_o$	$10^5 \times \sin^2\theta_c$	d(Å)	I	$10^5 \times \sin^2\theta_o$	$10^5 \times \sin^2\theta_c$	d(Å)	I	$10^5 \times \sin^2\theta_o$	$10^5 \times \sin^2\theta_c$
002	16·21	f	357	357	16·10	vvf	362	362	15·94	f	370	366
004	8·06	vf	1,443	1,429	—	—	—	1,448	7·96	vf	1,480	1,465
011	7·98	vf	1,475	1,483	7·91	f	1,501	1,499	7·84	f	1,528	1,519
012	7·34	ms	1,743	1,751	7·28	s	1,772	1,771	7·24	ms	1,789	1,794
101	—	—	—	1,852	—	—	—	1,848	—	—	—	1,823
102	6·65	ms	2,121	2,120	6·65	s	2,121	2,120	6·69	ms	2,096	2,098
013	6·53	vf	2,204	2,198	6·49	vf	2,230	2,223	6·44	w	2,262	2,252
103	6·05	w	2,563	2,567	6·03	w	2,584	2,572	6·05	w	2,563	2,556
014	5·75	vf	2,832	2,823	5·72	f	2,868	2,857	5·68	w	2,904	2,893
110	—	—	—	3,157	—	—	—	3,166	—	—	—	3,159
104	5·423	3,190°		3,191	5·410		3,205°	3,206	5·417		3,197°	3,197
006	↓	vs b	↓	3,215	↓	vs b	↓	3,258	↓	vs vb	↓	3,298
111	5·379		3,243°	3,246	5·353		3,274°	3,256	5·340		3,290°	3,251
112	5·181	vvf	3,495°	3,514	5·158	w	3,527	3,528	5·128	f b	3,568°	3,526
015	5·088	f	3,624	3,628	5·059	w	3,665	3,671	5·020	f b	3,723	3,717
113	—	—	—	3,960	—	—	—	3,981	—	—	—	3,984
105	4·854	m b	3,983	3,996	4·838	ms	4,009	4,020	4·828	m	4,026	4,021
114	4·520	s b	4,593	4,586	4·506	vs	4,620	4,614	4·520 →	ms vb	4,593° →	4,725
016	—	—	—	4,609	—	—	—	4,667	→4·449		→4,740°	4,725
106	—	—	—	4,977	—	—	—	5,016	4·314	vf	5,041	5,029
115	4·172	ms	5,390	5,390	4·157	s	5,430	5,429	4·168 →	ms b	5,400° →	5,449
									→4·134		→5,489°	
020	4·105	w	5,569	5,576	4·083	ms	5,629	5,635	4·054	m	5,710	5,710
021	—	—	—	5,666	—	—	—	5,725	—	—	—	5,802
008	—	—	—	5,715	—	—	—	5,792	—	—	—	5,862
017	4·039	f	5,751	5,772	4·011	vvf	5,832	5,843	3·990	f	5,894	5,915
022	3·969	vf	5,955°	5,933	3·952	vvf	6,007	5,997	—	—	—	6,077
107	3·905	vs	6,153	6,140	3·892	s	6,195	6,191	3·888 ↓	s b	6,207° →	6,220
									3·872 ↓		→6,258°	
116	3·836	m	6,375°	6,371	3·820	ms	6,429°	6,424	—	—	—	6,457
023				6,380				6,449	3·795	m b	6,514°	6,535
024	—	—	—	7,005	—	—	—	7,083	—	—	—	7,176
200	—	—	—	7,050	—	—	—	7,030	—	—	—	6,927
018	—	—	—	7,109	3·610	f b	7,198	7,200	—	—	—	7,290
201	3·627	m	7,131°	7,140	3·630	f b	7,119	7,121	3·656	f	7,019	7,018
120	—	—	—	7,339	—	—	—	7,392	—	—	—	7,442
202	—	—	—	7,408	3·558	vs	7,414°	7,392	3·585	w	7,300	7,293
121	3·554	m b	7,425°	7,428	—	—	—	7,483	↓	↓ b	↓	7,534
108	—	—	—	7,478	—	—	—	7,550				7,594
117	3·525	m	7,551	7,534	3·512	f	7,609	7,601	3·495	ms	7,679	7,648
122	—	—	—	7,696	—	—	—	7,754	—	—	—	7,809
025	—	—	—	7,809	—	—	—	7,897	—	—	—	8,000
203	—	—	—	7,854	—	—	—	7,845	—	—	—	7,751
123	3·393	vf	8,150	8,143	3·383	f	8,198	8,207	—	—	—	8,267
210	—	—	—	8,434	—	—	—	8,439	—	—	—	8,354
204	3·327	ms	8,475	8,480	3·327	s	8,475	8,478	3·347	s	8,378	8,392
211	—	—	—	8,534	—	—	—	8,530	—	—	—	8,446
019	—	—	—	8,627	—	—	—	8,739	—	—	—	8,847
124	3·269	ms	8,781	8,767	3·262	ms	8,818°	8,840	—	—	—	8,908
212				8,802				8,801	3·266	f	8,794°	8,721
026	—	—	—	8,791	—	—	—	8,893	3·228	f	9,005	9,007
00·10	3·242	f	8,930	8,934	—	—	—	9,050	—	—	—	9,160
109	—	—	—	8,996	—	—	—	9,088	—	—	—	9,151
213	—	—	—	9,248	—	—	—	9,254	—	—	—	9,179
205	3·179	ms	9,282	9,284	3·177	s	9,294	9,293	3·190	s	9,218	9,216

* s = strong, m = medium, w = weak, f = faint, b = broad line; ° = line omitted from least-squares calculation.

anhydrous forms and to get least-squares estimates of the cell dimensions of these forms (see Table 3). Table 2 shows the indexing and the agreement between $\sin^2\theta_{obs}$ and $\sin^2\theta_{calc}$ for the three forms.

Comparison of the powder photographs showed that:

(1) small progressive changes of cell parameters occur (*a* increasing, *b* and *c* decreasing) and that the volume decreases with loss of water.

(2) most lines become progressively less sharp as water is lost, indicating the onset of disorder and strain.

(3) all three patterns appear to be orthorhombic.

(4) a few lines change their relative intensities rather conspicuously, indicating appreciable rearrangement of the packing in at least parts of the molecule.

TABLE 3. CRYSTALLOGRAPHIC DATA FOR "WET," EQUILIBRATED AND ANHYDROUS δ-FORMS OF CEPHALORIDINE. (Cell parameters from least-squares calculations; standard deviations are quoted in brackets.) M_x is experimental value of molecular weight of asymmetric unit: M'_x is the value of M_x after scaling to make $M'_x = 433{\cdot}5$ for "wet" δ-form.

	"Wet"	Equilibrated	Anhydrous
a }	7·296(4)	7·306(2)	7·361(3)
b } (Å)	8·204(5)	8·161(3)	8·107(5)
c	32·407(14)	32·201(12)	32·008(24)
V (Å^3)	1940·9(1·7)	1920·1(1·1)	1910·1(2·1)
D_x (g. cm^{-3})	1·473(4)	1·470(4)	1·461(4)
M_x	430·5(1·2)	425·1(1·2)	420·3(1·2)
M'_x	433·5(1·2)	428·1(1·2)	423·3(1·2)
Expected M.	433·5(1H_2O)	429·0($\frac{1}{2}$ H_2O)	415·5(no H_2O)
No. of lines used in least squares calculation	20	22	18

Other photographs taken at intermediate stages of hydration gave intermediate features on the powder patterns, and both the "wet" and anhydrous forms on exposure eventually gave the pattern of the equilibrated form. The evidence suggests that the lattice changes are smooth and reversible.

The space group $P2_12_12_1$ has four equivalent general positions and no special positions, so that if the space group symmetry is adhered to strictly, the only permissible stoichiometries for the cell contents would be

$$4\ (\text{Cephaloridine}).4n(H_2O) \quad (n = 0,1,2\ldots).$$

A water content of 3 molecules per cell could only occur by random partial occupancy of water sites. The level of hydration can be checked only if both the cell densities and parameters are measured accurately enough. The densities were measured by flotation in a centrifuge in mixtures of trichloroethylene and carbon tetrachloride. The cell parameters quoted are all affected equally by a small correction for film shrinkage for which allowance has not been made; this has apparently led to values for cell volume being about $\frac{3}{4}\%$ too small and to a similar error in the magnitudes of the molecular weights, M_x. Scaling up the values so that the fully hydrated form has 4 molecules of water per cell gives the values, M'_x, in Table 3. The loss in passing from "wet" to equilibrated δ-form is $0{\cdot}30 \pm 0{\cdot}13$ mole (and is not, of course, affected by rescaling), but the anhydrous δ-form has too high a value of M_x; it corresponds to 0·43 mole of water. The density of this form was measured on a freshly prepared specimen under conditions that should prevent absorption of water. Nevertheless, it is likely that despite efforts to encapsulate and photograph the crystals without delay, water was absorbed during the exposure: a swelling of the lattice during the exposure may have contributed to the broadness of the lines for the anhydrous δ-form.

The crystallographic results in general corroborate the spectroscopic

conclusions, but indicate that loss of water must occur randomly from the water sites and that there cannot be a sharply defined, stable stoichiometry of $\frac{3}{4}$ mole of water. The deterioration of crystallinity, the progressive changes in cell size, the changes in relative intensity of some lines as water is lost and the apparently reversible nature of the changes suggest that the δ-form is a smoothly and reversibly swellable structure, in which there is some rearrangement of the packing (of some at least) of the cephaloridine molecules. Similar reversible variation in hydration, which ignores strict space group requirements, is known among zeolites (e.g., faujasite), clay minerals that swell in only one dimension (e.g., montmorillonite or vermiculite), and large organic molecules (e.g., vitamin B_{12} and proteins), but the number of water molecules is then much larger and considerable disorder occurs. Partial occupancy, defying space group requirements, is not rare when the number of solvated water molecules is low, but we have not so far found a clear precedent for it occurring as an equilibrium condition in a swellable lattice.

The density changes are proportional to the water content, but the change from "wet" to dry δ-form is only 0·7% compared with a weight loss of 4·3%. The major consequence of this loss of water is, therefore, a reduction of 3·6% in the cell volume, i.e., the molecules collapse to fill the holes vacated by the water.

The density is high for a material of this constitution, and consideration of ways of packing the molecules into the cell suggest that the packing must be tight. Presumably long-range electrostatic and intermolecular amide dipole-dipole forces and hydrogen bonding are responsible for the collapse of the structure in the absence of water. But, since the infrared spectra show no significant changes in the secondary amide hydrogen-bond frequencies on dehydration it can be deduced that the molecule is flaccid and offers little resistance to crumpling, i.e. the lattice can contract and the holes can be filled easily without significant alteration of the hydrogen-bonding distances. Changes of lattice parameter have little effect on the electrostatic and dipolar energies, so that there is little inducement to squeeze out the three molecules of water per cell.

But for the infrared evidence, the crystallographer, in the absence of a detailed knowledge of atomic positions and, in view of the sharpness of the powder lines, would have expected the extra water in the fully hydrated form to go into vacant sites crystallographically equivalent to those occupied by the other water molecules. The conclusion seems inescapable, that if in the "wet" δ-form structure all atoms had gone into strictly equivalent positions, the water molecules would have been unable to link effectively with the β-lactam carbonyl groups. Instead, a free-energy minimum has been achieved by accepting some measure of structural disorder. Roughly three in every four water molecules make effective links, but in so doing displace and distort the cephaloridine molecule somewhat (and probably themselves move from strictly equivalent sites), thus effectively closing the "hole" adjacent to the fourth β-lactam carbonyl group. The "extra" water is then loosely held and is easily squeezed out. Removal of water from the "$\frac{3}{4}$ mole hydrate" results in further crumpling and disorder.

The broadness of some of the lines for the anhydrous δ-form and the higher standard deviations of its cell parameters may represent an incipient departure from orthorhombic symmetry, but there is no clear evidence for this, nor is it evident how $P2_12_12_1$ might degrade.

Consideration has been given [on the lines considered by Dunitz (1964)] to the possibility that the orthorhombic symmetry might only be simulated by multiple twinning, but we find it difficult to see any explanation for the observed systematic absences.

We can only conclude that the whole process involves progressive random disorder from space group $P2_12_12_1$. The equilibrium is then thermodynamically controlled and represents a balance between the energies associated with (1) water attached to the β-lactam carbonyl group, and (2) the squeezing action of the electrostatic and dipolar forces. Presumably the equilibrium hydration level could be modified by temperature, pressure and ambient humidity changes. The crystallographic results corroborate all other findings, but indicate that the equilibrated δ-form is not a sharply defined stoichiometric entity as are most other hydrates. It has the composition 4 : 3 only by accident and within present experimental accuracy. From the poor quality of even the best single crystals examined it seems unlikely that a detailed study of its structure will be achieved.

Experimental

The infrared spectra (4,000–400 cm^{-1}) were recorded on a Perkin-Elmer model 521 spectrophotometer fitted with a diffraction grating. The compounds were examined as Nujol mulls mounted between potassium bromide plates. The mulls of hygroscopic forms of cephaloridine were prepared under anhydrous conditions in a dry-box; care was taken not to expose the mulls to a moist atmosphere. The cephaloridine samples satisfied the tests described by Martin & Shaw (1965) and were examined by pmr spectroscopy for solvent impurities, such as acetone, *NN*-dimethylacetamide, *NN*-dimethylformamide, dimethyl sulphoxide, ether, methanol and triethylamine. The pmr spectra of 10% solutions in heavy water were measured at 38° on a Varian Associates A60 Spectrometer at a sweep rate of 1 cycle/sec/sec and calibrated against sodium 3-(trimethylsilyl)-propane-1-sulphonate used as an internal standard. The X-ray powder photographs were taken on a Nonius Guinier (Mark 2) camera with either Cu- or Fe-Kα-radiation.

Water-content/vapour-pressure measurements were conducted at 17·5° on samples of cephaloridine contained in a closed, evacuated system in which the pressure of the water vapour could be measured directly on a simple manometer. Measurements were made by filling the apparatus (of known volume) with water vapour at its saturation pressure, allowing the cephaloridine crystals to absorb some of, and reach equilibrium with, the water vapour, and then recording the pressure. An adsorption isotherm was thus plotted. By a similar procedure step-wise desorption isotherms were also obtained.

The rate of intake of water vapour was measured by placing samples (about 4 g) of dried δ-form on a top-loading balance (Mettler P120) alongside suitable wicks arranged to give a substantially saturated atmosphere under a bell-jar that covered the pan area. A curve of weight-gain against time was plotted and showed a break at a point corresponding to approximately ¾ mole of water.

α-Form of cephaloridine. Triethylamine (2·04 ml) was added dropwise with stirring to a filtered solution of cephaloridine hydronitrate (6·89 g) (Eardley, Stocker & Long, 1966) in *NN*-dimethylacetamide (35 ml). After being kept at 4° for 4 hr, crystals of the α-form were filtered off, washed with *NN*-dimethylacetamide (10 ml) and acetone (3 × 25 ml), and dried at room temperature under reduced pressure. Prolonged drying at pressures down to 10^{-5}–10^{-6} mm did not reduce the *NN*-dimethylacetamide content of the sample below 0·05 mole, as indicated by the intensity of pmr peaks at τ6·92, 7·07 and 7·90.

β-Form of cephaloridine. The β-form of cephaloridine was prepared by a similar method to that used for the α-form except that *NN*-dimethylacetamide was replaced by *NN*-dimethylformamide; the intensity of the pmr peaks at τ6·97 and 7·13 indicated that the product contained about 0·1 mole of *NN*-dimethylformamide.

δ-Form of cephaloridine. The α-form of cephaloridine (14·5 g) was dissolved in water (40 ml) by warming and the filtered solution kept at room temperature until crystallization started. The solution was then stored for 16 hr at, and filtered at, 4°. The δ-form crystals were washed, in turn, with ice-water (4 × 10 ml) and acetone, and dried at 0·1 mm and room temperature over P_2O_5 overnight; this gave a product containing about ¾ mole of water. Anhydrous δ-form was obtained by drying the hydrated form at 40° at 0·01 mm over P_2O_5 for 6 days. On exposure to a normal laboratory atmosphere the anhydrous material picked up moisture and reverted to the equilibrium hydrate containing ¾ mole of water. Crystals containing about ¾ mole of heavy water were produced by exposing the anhydrous material to heavy water vapour in an otherwise evacuated system (0·1 mm) for 12 hr.

δ-Form of deuterated cephaloridine. The δ-form of cephaloridine was recrystallized from heavy water, washed with acetone and dried under reduced pressure to remove acetone. The deuterated product obtained in this way contained about ¾ mole of heavy water. Prolonged drying below 0·01 mm gave the anhydrous δ-form of deuterated cephaloridine, which on exposure to either water or heavy water vapour for 12 hr in an otherwise evacuated system (0·1 mm) gave crystals containing about ¾ mole of water or heavy water, respectively.

ε-Form of cephaloridine. The μ-form (see below) was dried *in vacuo* for 60 hr at room temperature to give the ε-form. The intensity of the

pmr peak at τ6·60 indicated that the product contained about 0·01 mole of methanol.

ζ-Form of cephaloridine. Triethylamine (0·8 ml) and a few drops of acetone were stirred into a filtered solution of cephaloridine hydronitrate (2 g) in dimethyl sulphoxide (15 ml). Acetone (90 ml) was added and, after 10 min, the ζ-form crystals were filtered, washed with acetone and dried for 2 hr at room temperature under reduced pressure. The pmr spectrum of the material was identical with that for the other forms except for a peak at τ7·25, the intensity of which indicated the presence of about 1 mole of dimethyl sulphoxide. Similar solvates were formed with ethylene glycol, diethylene glycol and benzyl alcohol.

μ-Form of cephaloridine. The preparation of the μ-form must be carried out in a dry-box. Triethylamine (1·0 ml) was added dropwise, with stirring, to a mixture of absolute methanol (25 ml) and a filtered solution of cephaloridine hydronitrate (2·5 g) in *NN*-dimethylacetamide (10 ml). A further 25 ml of methanol were added, and the white crystals of μ-form filtered, washed with methanol (20 ml) and acetone (2 × 20 ml), and dried. The intensity of the peak at τ6·60 in the pmr spectrum showed the presence of about one mole of methanol.

When the μ-form was exposed to a normal laboratory atmosphere, it reverted to δ-form containing about $\frac{3}{4}$ mole of water; this process was reversed by exposing the hydrate to dry methanol vapour. Shaking the μ-form with liquid *NN*-dimethylacetamide, *NN*-dimethylformamide or dimethyl sulphoxide at 4° for 16 hr gave the α-, β- or ζ-forms, respectively. Shaking these forms with methanol under the same conditions gave the μ-form.

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The use of the Coulter Counter for the particle size analysis of some emulsion systems

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The Coulter Counter is suitable for the particle size analysis of emulsions containing insoluble oils. All the emulsions obey the log normal distribution of particle size if allowance is made for particles below the lower limits of the apparatus. The variation of particle size with emulsifier concentration and volume fraction has been studied for liquid paraffin emulsions stabilized by potassium laurate and potassium arabate.

IN an investigation to examine the effect of emulsifier concentration on the rheology of liquid paraffin emulsions stabilized by potassium laurate and potassium arabate (Davis, 1967), it was necessary to measure the particle size distribution.

The Coulter Counter has been used to study a wide variety of particulate systems, including emulsions, since its introduction in 1956. Comparisons with other size analysis methods have been made by Irani (1960) for glass beads, Batch (1964) for coal dust and by Marshall & Taylor (1965) and Groves, Scarlett & Freshwater (1966) for emulsions. Schrenzel (1966) has discussed the errors inherent in the microscopic methods of analysis of emulsions and has concluded that the Coulter Counter, with its high statistical accuracy, is the preferable instrument. However there are still a number of problems that are raised by the literature.

Higuchi, Okada & Lemberger (1962), Samyn & McGee (1965) and Groves (1966) found that initial dilution of an emulsion with saline had no detectable effect on particle size distribution, whereas Marshall & Taylor (1965) and Schrenzel (1966) reported that direct saline dilution was detrimental. This may be due to differences in the emulsifying agents and disperse phases employed.

The use of electrolyte containing emulsifier, at concentrations below the critical micelle concentration (CMC) has been described by Rowe (1965) and Samyn & McGee (1965) although no explanation was given for its addition. Groves (1966) advocated the use of surfactant at concentrations above the CMC to prevent aggregation of the emulsion by saline.

Nash (1965) considered that the Coulter Counter was unsuitable for aggregated systems as these would be broken down on dilution. Higuchi & others (1962), however, used the instrument as a direct means of studying the aggregation and disaggregation of hexadecane emulsions stabilized by Aerosol O.T. (Cyanamid Ltd). Swift & Friedlander (1964) used the instrument to study the coagulation of hydrosols.

Emulsion systems that have been studied have all had insoluble oils as disperse phase. It is a matter of conjecture whether emulsions of soluble oils stabilized by thick interfacial films of the hydrophilic colloid type (Shotton & White, 1963) can be counted.

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* This work formed part of a thesis by S.S.D. for the degree of Ph.D. in the University of London.

The representation of the size distributions of emulsions by smooth continuous mathematical functions has been advocated by Becher (1965). The distribution can then be easily defined and an estimate made of those particles that are outside the limits of the sizing apparatus. Empirical distribution functions have been suggested by Rossi (1933) and Jellinek (1950) among others. The theoretical work of Rajagopal (1959a) and the observations of Epstein (1947, 1948) indicate that the particle size distribution of an emulsion formed by disrupture processes should be log normal. Many workers have since found that the log normal distribution (eqn 1) is a valid mathematical model for describing the size distributions of particulate materials (Rajagopal, 1959b; Batch, 1964; Talman, Davies & Rowan, 1967)

$$y = \frac{1}{\ln \sigma \sqrt{2\pi}} \exp \left[- \frac{(\ln x - \ln M)^2}{2 \ln^2 \sigma} \right] \quad \ldots \quad (1)$$

where y is the probability function, x the particle diameter, σ the standard deviation and M the geometric mean particle size.

Other modifications of the normal distribution have also been used (Schwarz & Bezemer, 1956; Mugele & Evans, 1951; Sprow, 1967). The log normal distribution has been described in detail by Kottler (1950) and Smith & Jordan (1964). Truncated log normal distributions may be converted to their original distributions by algebraic and statistical methods (Kottler, 1950; Rajagopal, 1959b) or by graphical methods (Irani & Callis, 1963).

Using the Coulter Counter, Rowe (1965), Marshall & Taylor (1965) and Schrenzel (1966) reported a poor fit to the log normal distribution. However, these workers may not have considered particles outside the limits of the apparatus (Batch, 1964) or the inherent statistical errors of the distribution (Smith & Jordan, 1964; Gwyn, Crosby & Marshall, 1965).

These problems have been investigated in the present work so as to enable the determination of particle size distributions for potassium laurate and potassium arabate emulsions of liquid paraffin.

Experimental

Materials. Liquid paraffin of British Pharmacopoeia quality. Benzene of Analar quality. Lauric acid of 99% purity was checked by gas chromatography and potassium arabate prepared according to Shotton (1955).

Apparatus. Particle size measurements were made using a Coulter Counter model A, industrial, with 30 and 100 μ orifice tubes previously calibrated with polyvinyl toluene lattices and pollens of known diameter. Details of the apparatus and its calibration have been given by Wachtel & LaMer (1962), Edmundson (1967) and Allen (1967).

PREPARATION OF EMULSIONS

The required quantities of oil and surfactant solution were weighed into a stainless steel beaker and an initial dispersion brought about by means of a high speed stirrer fitted with a "dispersator" head. Further

homogenization was produced by an Omerod Q.P. hand homogenizer. A total of 4 passages was made with the homogenization pressure increased each time. To obtain a standardized homogenization procedure, the same series of arbitrary pressures were used for each emulsion system.

The emulsions were stored at 25° for four days before examination. Formulation details are given in Table 1.

PROCEDURE

The electrolyte was commercial normal saline, low in particulate contamination. The emulsions were diluted (1 : 250,000) with saline using a two step process to give a suitable concentration for counting (Samyn & McGee, 1965) and counted immediately. The effect of diluting the

TABLE 1. FORMULATION DETAILS AND PARTICLE SIZE RESULTS POTASSIUM LAURATE-LIQUID PARAFFIN EMULSIONS

Emulsifier conc. % w/w	ϕ	Particle size data: Mean vol. diam. μ	s.d. σ	Correl. coeff.	Conc. % w/w	ϕ	Particle size data: Mean vol. diam. μ	s.d. σ	Correl. coeff.
Laurate									
10·0	0·05	2·77	2·80	0·998	1·0	0·05	3·50	2·52	0·998
	0·11	2·56	2·57	0·991		0·11	3·98	2·45	0·997
	0·16	3·10	2·87	0·996		0·16	3·34	2·49	0·995
	0·22	2·20	2·28	0·997		0·22	3·92	2·19	0·995
	0·33	3·15	2·47	0·997		0·33	3·97	2·24	0·996
	0·43	2·09	2·37	0·991		0·43	4·26	2·27	0·988
	0·53	2·68	2·24	0·991		0·53	3·39	2·02	0·997
	0·63	2·40	2·19	0·997		0·63	3·69	2·23	0·995
5·0	0·05	2·95	2·55	0·987	0·75	0·05	4·10	2·34	0·995
	0·11	3·62	2·54	0·996		0·11	4·00	2·34	0·997
	0·16	2·88	2·88	0·995		0·16	3·44	2·05	0·992
	0·22	3·06	2·31	0·976		0·22	4·17	2·23	0·995
	0·33	3·35	2·11	0·995		0·33	3·29	1·95	0·996
	0·43	2·81	2·16	0·994		0·43	3·92	2·16	0·993
	0·53	3·01	2·09	0·998		0·53	3·83	2·13	0·997
	0·63	3·61	2·35	0·997		0·63	3·78	2·21	0·997
2·5	0·05	3·00	2·60	0·998	0·5	0·11	5·12	2·43	0·995
	0·11	3·98	2·45	0·997		0·22	5·08	2·22	0·995
	0·16	3·04	2·52	0·996		0·33	4·45	2·10	0·995
	0·22	4·07	2·31	0·997		0·43	5·31	2·18	0·997
	0·33	3·52	2·37	0·997		0·53	4·49	2·19	0·997
	0·43	3·41	1·99	0·991		0·63	5·06	2·09	0·997
	0·53	3·28	2·20	0·999					
	0·63	3·36	2·19	0·998					
Arabate									
0·75	0·11	4·66	2·12	0·992	8·0	0·12	3·78	2·28	0·989
	0·22	5·04	2·23	0·993		0·23	5·27	2·05	0·986
	0·33	3·57	3·76	0·976		0·33	9·91	2·28	0·985
	0·43	8·35	4·35	0·991		0·44	10·3	1·65	0·998
	0·53	21·5	2·01	0·991		0·54	13·6	1·62	0·998
	0·63	28·3	2·13	0·995		0·64	16·8	1·74	0·987
2·25	0·11	3·84	2·26	0·994	12·0	0·12	4·61	2·24	0·993
	0·22	4·84	2·26	0·989		0·23	4·88	2·10	0·988
	0·33	6·31	2·09	0·996		0·34	9·46	2·01	0·982
	0·43	13·8	1·54	0·951		0·44	11·0	1·72	0·996
	0·53	14·5	1·62	0·996					
	0·63	17·0	1·75	0·990					
4·5	0·11	4·02	2·38	0·981					
	0·22	6·04	2·18	0·994					
	0·33	5·89	2·19	0·991					
	0·43	13·5	1·60	0·991					
	0·53	12·3	1·63	0·990					
	0·63	13·6	1·70	0·998					

emulsion with saline was investigated by making initial dilutions with either saline or distilled water. No significant difference in particle size results was detected.

The addition of surfactant (potassium laurate) at concentrations below the CMC appeared to have no effect on particle size distribution but did create counting and filtration difficulties due to the formation, by carbon dioxide, of small needle shaped crystals of acid soap. Concentrations above the CMC, as suggested by Groves (1966), were not employed for, besides filtration and calibration problems, such a small quantity of oil reaches the counting vessel that loss of the smaller particles may well occur due to solubilization (McBain & Richards, 1946).

RESULTS

These are in Table 1.

DATA ANALYSIS

The raw count, after correction for coincident passages, gives the number of particles, N_i, that have volumes greater than V_i, for the range $i = 1$ to $i = n$.

V_i is related to the threshold value, T_i, of the instrument and the particle diameter (d_i) may be calculated from

$$d_i = KT_i^{1/3} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where K is the calibration constant.

For the size range d_i to d_{i+1} the mean volume is proportional to $\bar{T}_i$ where

$$\bar{T}_i = \tfrac{1}{2}(T_i + T_{i+1}) \quad .. \quad .. \quad .. \quad (3)$$

and the fraction of the total volume of particles (Q_i) within the interval is

$$Q_i = (N_i - N_{i+1})\bar{T}_i \quad .. \quad .. \quad .. \quad (4)$$

If d_n is the smallest size level of the instrument and d_z the size of the smallest particle, then in the analysis of the results two cases may arise.

(i) If d_n is *below* the size of the smallest particle (d_z) the complete distribution is obtained by calculating the percentage volume of the particles above a given size (% volume oversize) C_i

$$C_i = \frac{\sum_{i}^{i} Q_i \times 100}{\sum_{i}^{n} Q_i} \quad .. \quad .. \quad .. \quad (5)$$

(ii) In many cases the size of the smallest particle (d_z) is below the limit of the apparatus. For such systems the Coulter Counter handbook (1966) suggests a graphical extrapolation method to find N_z using a plot of log N_i against log T_i. This is an empirical and rather lengthy procedure only suitable for cases where the log N_i versus log T_i plot is curved. Extrapolation to a low value of log T_i, (log T_z) where log N_i is independent of log T_i, gives the log N_z value.

then $$Q_z = (N_z - N_n)\frac{(T_z - T_n)}{2} \quad .. \quad .. \quad (6)$$

and

$$C_i = \frac{\sum_{1}^{i} Q_i \times 100}{\sum_{1}^{z} Q_i} \quad .. \quad .. \quad .. \quad (7)$$

A log probability plot of C_i against log d_i for a suitable emulsion (amenable to the Coulter handbook method) is shown in Fig. 1, curve A.

For many emulsion systems the plot of log N_i versus log T_i does not show curvature and no extrapolation is possible. If the particle size distribution is assumed to be log normal, as predicted by Rajagopal (1959a), a more rigorous procedure, amenable to computer analysis, can be developed for the present work. If the distribution is considered to have been truncated at the smallest measured size (0·9 μ for the 30 μ tube), i.e., no particles below d_n, then calculation of the C_i values using equation (5) gives the curve B, Fig. 1. This is typical of a truncated log normal distribution with an asymptote to the size at which truncation has occurred (0·9 μ). Truncated log normal distributions of this type may be corrected to their original distribution (Irani & Callis, 1963) by extrapolation of the straight line obtained at higher levels to the asymptote size (0·9 μ). The value of probability at this point P gives the percentage oversize obtained had there been no truncation. Multiplication of C_i values by P/100 gives a corrected log normal distribution that takes into account those particles below the limit of the apparatus. Using the same emulsion as before, a close agreement in C_i values is obtained with the Coulter handbook method, Fig. 1, curve C.

The values of percent oversize obtained at the largest sizes were subject to statistical scatter in some cases and for this reason the extrapolation considered those points lying between the limits of 20–80% probability (Kottler, 1950; Smith & Jordan, 1964).

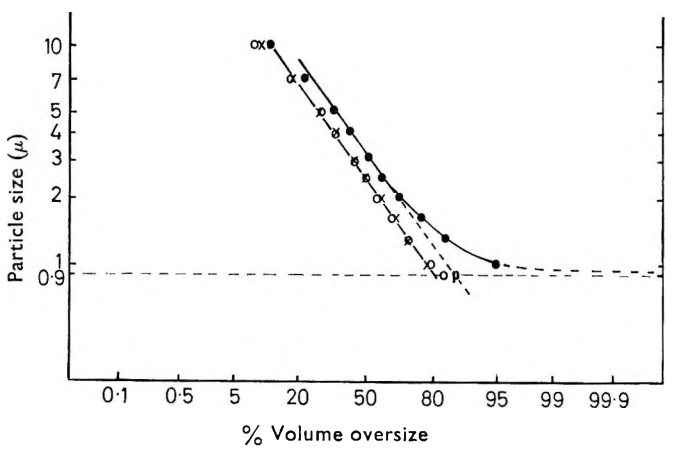

FIG. 1. The log normal distribution and its correction for instrumental truncation. Abscissa: Percentage volume oversize (on probability scale) (C_i). Ordinate: Particle size (μ) (log scale) (d_i). × Curve A, Coulter handbook extrapolation. ● Curve B, original uncorrected curve. ○ Curve C, original curve corrected for instrumental truncation.

Complete data analysis was carried out using the University of London Atlas computer and the resultant corrected log normal curve analysed statistically. The following were evaluated: (i) the correlation coefficient for a linear regression analysis on the corrected log probability plot; (ii) the mean volume diameter; (iii) the standard deviation. Once (ii) and (iii) have been evaluated other parameters such as mean weight diameter, mean number diameter, specific surface area, polydispersity, etc., can be determined if required (Irani & Callis, 1963; Rowe, 1965).

POTASSIUM LAURATE–LIQUID PARAFFIN EMULSIONS

These emulsions provided suitable systems for counting. Above 1·0% soap the emulsions were aggregated (Shotton & Davis, 1967) but microscopic examination showed that the aggregates were broken down by the two-step dilution for counting and the individual particles sized. The three typical results in Fig. 2 demonstrate the very good fit to the

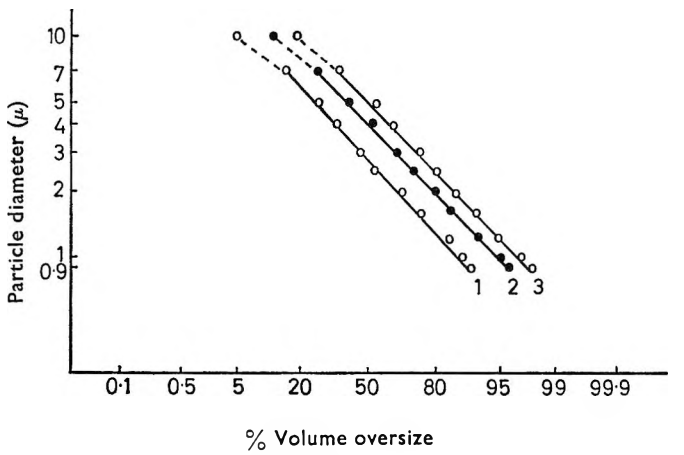

FIG. 2. The particle size distributions for three liquid paraffin, potassium laurate emulsions (log probability plot). Soap concentration 1, 10%; 2, 2·5%; 3, 0·5%; volume fraction 0·22.

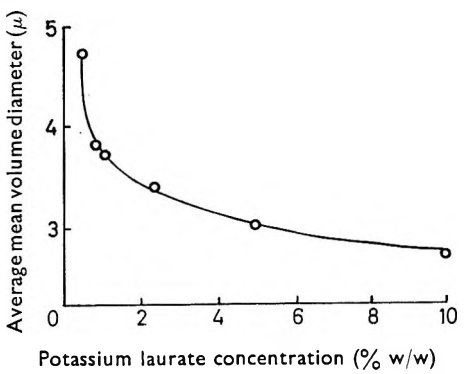

FIG. 3. The change in mean volume diameter with soap concentration for liquid paraffin, potassium laurate emulsions (average results for volume fractions 0·05–0·63).

log normal obtained after correction for instrumental truncation. In Table 1 only three of the potassium laurate, liquid paraffin emulsions give a linear regression coefficient of less than 0·990.

The mean volume diameter appears to be almost independent of volume fraction (ϕ), although in some cases a slight fall in size with increased ϕ is indicated. The average mean volume diameter (volume fractions 0·05 to 0·63) varies in an approximately exponential manner with soap concentration (Fig. 3).

POTASSIUM ARABATE–LIQUID PARAFFIN EMULSIONS

Microscopic examination showed that these emulsions were aggregated at high volume fraction (Davis, 1967) but the aggregates were *not* broken down on dilution. Both the aggregated and non-aggregated emulsions could be described by the log normal distribution, but with a generally lower value for the correlation coefficient than the laurate emulsions. A plot of average mean volume diameter (arabate concentrations 2·25 to 12·0%) against volume fraction demonstrates the aggregation of the emulsions when the *volume fraction* is in the region of 0·33 (Fig. 4).

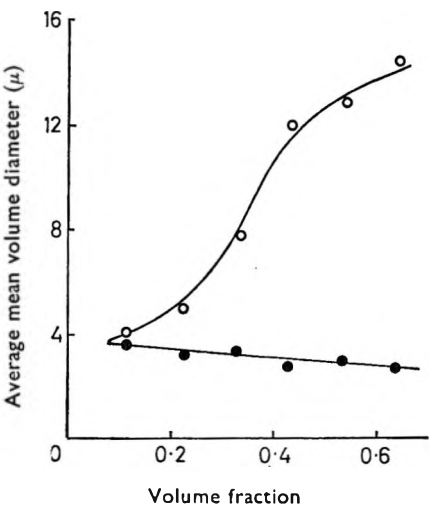

FIG. 4. The change in mean volume diameter with volume fraction for potassium arabate and potassium laurate emulsion of liquid paraffin (average results for arabate concentrations 2·25–12·0%, laurate concentration = 5%.) ○, Arabate, ●, Laurate.

Laurate and arabate emulsions stabilized by benzene could not be counted. At the high dilution used the benzene rapidly dissolved in the saline and only the background count for the saline was obtained. This effect was *not* prevented by the thick interfacial film produced by potassium arabate. Saturation of the saline with benzene enabled counts to be made but their accuracy was suspect due to a very high background count caused by small benzene droplets that were difficult to remove by filtration.

Discussion

Only emulsions with insoluble oils can be counted using the Coulter Counter. These include those emulsions stabilized by a thick interfacial film. Liquid paraffin emulsions aggregated by soap are completely broken down on dilution whereas arabate aggregates are not. Higuchi & others (1962), however, were able to count soap-stabilized hexadecane aggregates and it therefore appears that dilution breakdown depends both on the disperse phase and the emulsifier.

Volume fraction appears to have little effect on the mean particle diameter of liquid paraffin–potassium laurate emulsions. This is in contrast to Groves & Freshwater (1967) who found an increase in mean size with increased volume fraction. However their emulsions were stabilized by a cetrimide, cetostearyl alcohol complex which may explain the difference between the results. The mean volume diameter is related almost exponentially to the emulsifier concentration. Rowe (1965) suggested that the activity of the surfactant was the determining factor for particle size and that it was related to concentration by a log function. This is somewhat doubtful for Kolthoff & Johnson (1948) have shown that the activity of soaps is independent of concentration above the CMC, and an alternative explanation is therefore advanced.

THE EFFECT OF EMULSIFIER CONCENTRATION ON PARTICLE SIZE

Above the CMC the interfacial tension is almost constant (Powney & Wood, 1941) so that standardized homogenization of emulsions containing different soap concentrations will give, *initially*, globules of similar particle size distributions. The *resultant* globule sizes will depend on how quickly the surfactant reaches the interface and forms an interfacial layer sufficient to prevent coalescence. Ward & Tordai (1946) studied the diffusion of surfactants to the interface and obtained the equation

$$M = \int_0^t \left(\frac{dm}{dt}\right)_{x\to 0} dt = 2C\left(\frac{Dt}{\pi}\right)^{\frac{1}{2}} \quad .. \quad .. \quad (8)$$

where M is the surface concentration at time t, C the constant bulk concentration, x the distance from the interface and D the diffusion coefficient.

Assuming that a given surface concentration is required to prevent droplet coalescence then the time for that excess to be obtained, i.e., the time available for coalescence, will be proportional to the square of the reciprocal of the emulsifier concentration $\left(\frac{1}{C^2}\right)$

i.e.,
$$t = \frac{\pi M^2}{4DC^2} \quad .. \quad .. \quad .. \quad (9)$$

If the resultant globule sizes are proportional to the time available for coalescence then the mean globule size ($\bar{d}$) is proportional to $^1/C^2$.

This type of relation gives a plot somewhat similar to an exponential plot and gives a far better explanation of the shape of the curve in Fig. 3 than that advanced by Rowe (1965).

The increased viscosity of the continuous phase with emulsifier concentration will reduce the rate of coalescence and will have a similar, but slight, effect on the shape of the mean volume diameter, emulsifier concentration relation.

The aggregation of potassium arabate emulsions with volume fraction has been investigated in detail (Davis, 1967) and is due to a macromolecular bridging mechanism of the type described by Smellie & LaMer (1958) and discussed recently by Overbeek (1966).

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The influence of emulsifier concentration on the rheological properties of an oil-in-water emulsion stabilized by an anionic soap

E. SHOTTON AND S. S. DAVIS*

The rheological properties of liquid paraffin emulsions stabilized by potassium laurate have been found to be markedly dependent on emulsifier concentration. Reversible aggregation of the emulsion began at 1·0% soap and the more highly aggregated systems demonstrated pseudoplastic flow. A maximum relative viscosity was found at 5·0% soap concentration. The viscosity was dependent on particle size but correction for particle size differences did not alter the viscosity concentration relation. The addition of electrolytes to the emulsion demonstrated the difference between surfactant aggregation and electrolyte aggregation. The latter was explained by the Derjaguin-Verwey-Overbeek theory of colloid stability, whilst the former was only satisfactorily explained by the theory advanced by Cockbain in 1952 involving polymolecular adsorption at the oil-water interface and hydrophobic bonding.

THE influence of the emulsifier concentration on the flow properties of emulsions has not been extensively examined. Earlier work by Wilson & Parkes (1936) and Toms (1941), who used Ostwald U-tube viscometers, showed that the relative viscosity increased with increased emulsifier concentration. Similar results were obtained by Lawrence & Rothwell (1957) who examined concentrated oil in water emulsions at low shear rates. In a series of papers, Sherman (1950, 1955, 1963) investigated the effect of emulsifier type and concentration on the viscosities of oil in water and water in oil emulsions for non-ionic agents. The emulsifying agent concentration influenced the volume fraction at which inversion of the emulsion occurred and the maximum viscosity before inversion. Sherman (1960, 1963) advocated that the precise influence exerted by the emulsifier concentration on viscosity could be assessed only when all the emulsions had the same globule size and size distribution.

Two empirical expressions relating emulsifier concentration and viscosity were derived by Sherman (1959). At constant high volume fraction of disperse phase the expression

$$\eta_{rel} = e^{ac+b}$$

was suitable. Where η_{rel} is the relative viscosity, c the emulsifier concentration as % w/w of the total emulsion and a and b are constants. A second expression in the form

$$\ln \eta_{rel} = ac\phi + b$$

was found to hold over a range of volume fractions (ϕ).

The absence of a detailed investigation on oil-in-water emulsions stabilized by anionic agents has lead to the present work.

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* This work formed part of a thesis for the degree of Ph.D. in the University of London.

Experimental

MATERIALS

Liquid paraffin (B.P.). Lauric acid (99%) which was checked for purity by gas chromatography. Potassium hydroxide and potassium salts of Analar quality. Distilled water from an all glass still.

APPARATUS

Particle size measurement. A Coulter Counter, model A industrial, was used to size the emulsion systems employing a 30 μ aperture tube, calibrated with polystyrene lattices. The analysis of the results from the Coulter Counter measurements has been described by Shotton & Davis (1968).

Viscometry. A Couette viscometer was used which was basically that described by Perrin & Saunders (1966). Different bob and wire combinations allowed measurements to be made over the viscosity range of 0·8 to 80 centipoises. The apparatus was calibrated against suitable Newtonian liquids of known viscosity.

Microelectrophoresis. The charge on the oil globules was determined using a cylindrical microelectrophoresis cell based on the design of Bangham, Heard & others (1958). All measurements were made at 25·0° at the stationary level (zero electro-osmotic flow) at a field strength of 3·3 V cm^{-1}.

PROCEDURE

Formulation details are given in Table 1. Emulsions were prepared from weighed quantities of liquid paraffin and potassium laurate solution by initial dispersion with a dispersator and then passage through a hand homogenizer. Four days storage at 25° were allowed for interfacial equilibrium and the escape of air bubbles. The emulsion was then examined using microelectrophoresis, the Coulter Counter and the Couette viscometer.

Results

VISCOSITY

Table 1 summarizes the experimental results.

The emulsions were examined over the shear rate range of 0 to 120 sec^{-1}, the lowest shear rate measured being in the region of $2 \times 10^{-1} sec^{-1}$. Both Newtonian and non-Newtonian types of flow were exhibited but in all cases the flow curves passed through the origin. There was no evidence of time effects and the non-Newtonian flow curves were pseudoplastic in form. The analysis of the Newtonian flow curves was simple, as the gradient of the flow curve gave unique representation. Pseudoplastic flow was analysed by the following methods:

(i) The limiting viscosity calculated from the reciprocal of the slope of the straight line produced at higher shear rates (when all structure had been broken down) (η_{lim}).

(ii) The apparent viscosity at a given highest shear rate (120 sec^{-1}) calculated from the ratio of shear stress to shear rate (η_{app}).

(iii) The power law equation used originally by Farrow, Lowe & Neale (1928) and more recently by Scott Blair (1965)

TABLE 1. POTASSIUM LAURATE LIQUID PARAFFIN EMULSIONS FORMULATION DETAILS, VISCOSITY AND PARTICLE SIZE RESULTS

Laurate conc., % w/w	ϕ	Relative viscosity		Cross equation		n from power law eqn	Particle size	
		η_{rel}^{lim}	η_{rel}^{app}	η_0 centi-poises	α		Mean vol. diam., μ	s.d., σ
10·0	0·05	1·40	—	—	—	—	2·77	2·80
	0·11	1·92	2·03	—	—	—	2·56	2·57
	0·16	2·25	2·55	—	—	1·15	3·10	2·87
	0·22	3·14	3·88	24·3	—	1·30	2·20	2·28
	0·33	5·11	6·01	40·3	—	1·48	3·15	2·47
	0·43	7·86	12·5	170·2	0·0068	1·63	2·09	2·37
	0·53	13·2	21·9	291	0·0075	2·10	2·68	2·24
	0·63	23·5	44·5	653	0·0050	2·16	2·40	2·19
5·0	0·05	1·41	—	—	—	—	2·95	2·55
	0·11	1·86	1·96	—	—	—	3·62	2·54
	0·16	2·75	3·32	—	—	1·41	2·88	2·88
	0·22	3·31	4·11	28·3	—	1·51	3·06	2·31
	0·33	5·89	6·77	41·3	—	1·60	3·35	2·11
	0·43	10·9	14·7	184	0·0073	1·71	2·81	2·16
	0·53	23·8	31·3	330	0·0089	2·01	3·01	2·09
	0·63	44·0	72·3	840	0·0084	2·24	3·61	2·35
2·5	0·05	1·45	—	—	—	—	3·00	2·60
	0·11	1·80	2·10	—	—	—	3·98	2·45
	0·16	2·20	2·61	—	—	—	3·04	2·52
	0·22	2·75	3·24	5·82	—	1·08	4·07	2·31
	0·33	5·54	6·79	32·8	—	1·21	3·52	2·37
	0·43	8·15	15·1	72·5	0·0035	1·52	3·41	1·99
	0·53	15·0	22·3	219	0·0060	1·65	3·28	2·20
	0·63	23·3	45·8	360	0·0069	1·90	3·36	2·19
1·0	0·05	1·25		These systems exhibited Newtonian flow			3·50	2·52
	0·11	1·42					3·98	2·45
	0·16	1·72					3·34	2·49
	0·22	1·98					3·92	2·19
	0·33	2·75					3·97	2·24
	0·43	6·06					4·26	2·27
	0·53	12·5					3·39	2·02
	0·63	23·3					3·69	2·23
0·75	0·05	1·30					4·10	2·34
	0·11	1·45					4·00	2·34
	0·16	1·65					3·44	2·05
	0·22	2·02					4·17	2·23
	0·33	2·97					3·29	1·95
	0·43	5·32					3·92	2·16
	0·53	10·6					3·83	2·13
	0·63	22·0					3·78	2·21
0·5	0·11	1·49					5·12	2·43
	0·22	1·98					5·08	2·22
	0·33	3·03					4·45	2·10
	0·43	5·17					5·31	2·18
	0·53	9·53					4·49	2·19
	0·63	20·85					5·06	2·09

s.d. = standard deviation, log normal distribution.

$$\sigma^n = K\dot{\gamma}$$

where σ is the shear stress, $\dot{\gamma}$ the shear rate and K and n constants. The equation gives a simple mathematical representation of the pseudoplastic flow curves and provides a comparative constant n. The rheological significance of such double log plots is a matter of argument (Reiner, 1960, Scott Blair, 1965).

(iv) The method of Cross (1965) using the equation

$$\eta = \eta_\infty + (\eta_0 - \eta_\infty)/(1 - \alpha\dot{\gamma}^{2/3})$$

where η_0 is the limiting viscosity at zero shear, η_∞ the limiting viscosity at infinite shear and α a constant associated with the rupture of linkages.

At lower shear rates a plot of $1/\eta$ against $\dot{\gamma}^{2/3}$ gave a straight line from which the constants η_0 and α could be evaluated.

Systems containing 1·0% or less of soap were Newtonian in their flow properties (1·0% showed slight aggregation) whereas those with a greater quantity of soap were highly aggregated and demonstrated pseudoplastic flow.

For soap concentrations greater than 1·0% the relation between relative

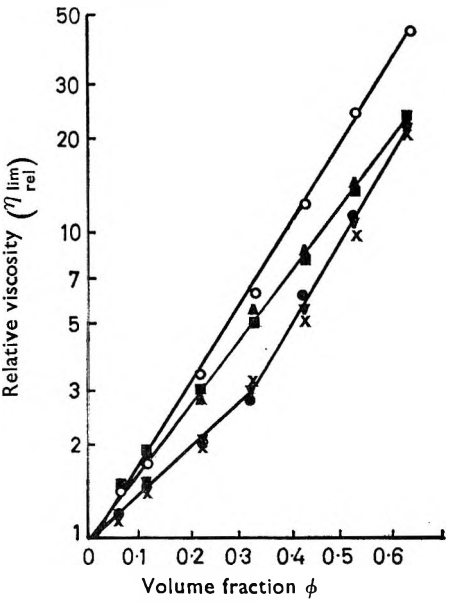

FIG. 1. The change in relative limiting viscosity with volume fraction for liquid paraffin-potassium laurate emulsions of different soap concentrations (log plot). Concentration of soap % w/w ■ 10·0, ○ 5·0, ▲ 2·5, ● 1·0, ▼ 0·75, × 0·5.

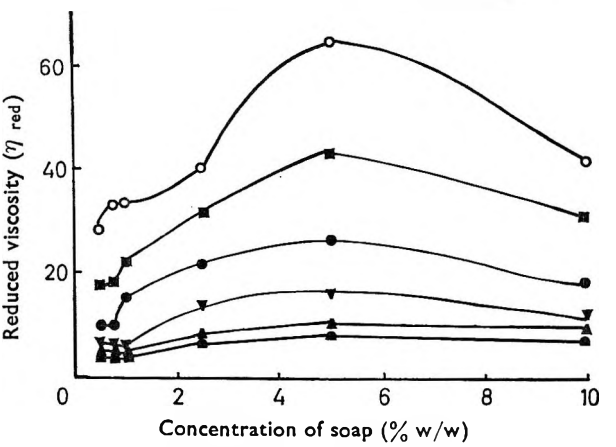

FIG. 2. The change in reduced viscosity with soap concentration for liquid paraffin-potassium laurate emulsions of different volume fraction. Volume fraction ○ 0·63, ■ 0·53, ● 0·43, ▼ 0·33, ▲ 0·22, ◠ 0·11.

limiting viscosity and volume fraction (Fig. 1) could be represented by the Richardson (1933) equation

$$\eta_{rel}^{lim} = e^{k\phi}$$

The systems of lower soap concentration gave a linear relation that showed a change of gradient at a volume fraction of 0·34 to 0·37.

The exponent n from the power law relation shows an approximately linear relation with volume fraction, with n having a maximum value for those systems containing 5·0% of soap.

To compare the results at different volume fractions on the same scale, they are expressed in Fig. 2 as reduced viscosity against soap concentration where the reduced viscosity

$$\eta_{red} = (\eta_{rel}^{lim} - 1)/\phi$$

Different volume fractions all show the same type of curve. The viscosity increases from 1·0% to a maximum at 5·0% soap and then falls.

TABLE 2. THE EFFECT OF PARTICLE SIZE ON THE VISCOSITY OF LIQUID PARAFFIN EMULSIONS

Laurate conc., % w/w	ϕ	H*	Relative viscosity η_{rel}^{lim}	Relative viscosity η_{rel}^{app}	Mean vol. diam. μ	s.d., σ	Laurate conc., % w/w	ϕ	H*	Relative viscosity η_{rel}	Mean vol. diam. μ	s.d., σ
10·0	0·22	1	2·68	2·80	5·51	2·71	1·0	0·22	1	2·52	6·01	2·23
		2	2·75	2·92	4·75	2·62			2	2·45	5·35	2·25
		3	2·83	3·10	4·20	2·76			3	2·25	4·50	2·15
		4	2·95	3·40	3·41	2·66			4	2·23	4·10	2·16
	0·43	1	6·46	8·47	5·52	2·45		0·43	1	6·25	5·95	2·15
		2	7·08	9·32	4·52	2·22			2	6·00	5·35	2·15
		3	7·30	9·95	4·06	2·43			3	5·95	4·80	2·24
		4	7·61	11·05	3·42	2·52			4	5·75	4·45	2·02
	0·63	1	17·4	20·9	6·75	2·42		0·63	1	24·0	6·11	2·02
		2	19·6	24·2	5·49	2·52			2	24·6	5·60	2·06
		3	20·4	26·3	4·51	2·41			3	24·9	4·73	2·04
		4	22·5	30·4	4·02	2·44			4	24·8	4·20	2·00
5·0	0·22	1	2·70	2·85	5·58	2·27	0·75	0·22	1	2·40	6·25	2·16
		2	2·81	2·90	5·30	2·24			2	2·45	5·50	2·21
		3	2·95	3·15	4·55	2·26			3	2·21	4·61	2·16
		4	3·20	3·61	3·65	2·30			4	2·15	3·72	2·19
	0·43	1	6·52	8·05	6·00	2·29		0·43	1	6·98	7·03	2·01
		2	7·20	8·95	5·56	2·26			2	5·99	5·51	1·99
		3	7·51	10·1	4·35	2·35			3	5·60	4·80	2·12
		4	7·83	11·0	4·02	2·32			4	5·38	4·10	2·12
	0·63	1	20·4	22·2	6·12	2·14		0·63	1	20·2	8·13	2·06
		2	22·1	29·3	5·34	2·21			2	20·9	5·98	2·12
		3	28·0	40·2	4·49	2·14			3	21·4	5·51	2·00
		4	31·2	48·1	3·99	2·17			4	21·8	5·50	2·08
2·5	0·22	1	2·53	2·96	5·50	2·51	0·5	0·22	1	—	6·78	2·28
		2	2·58	2·95	4·83	2·41			2	1·95	5·15	2·22
		3	2·62	3·05	4·45	2·43			3	1·75	4·85	2·16
		4	2·65	3·15	4·00	2·50			4	1·78	4·79	2·26
	0·44	1	6·10	7·68	6·02	2·12						
		2	6·22	7·75	5·11	2·14						
		3	6·38	8·33	4·53	2·20						
		4	6·52	8·81	4·01	2·12						
	0·63	1	21·1	26·2	5·61	2·12						
		2	24·2	30·5	4·80	2·04						
		3	25·8	34·2	4·35	2·00						
		4	28·8	38·4	3·80	2·00						

*H = No. of times homogenized.
s.d. = standard deviation, log normal distribution.

The results calculated from the equation of Cross (1965) show that the greatest η_0 and α are also to be found at 5·0% (Table 1). The α values derived are much lower than those obtained by Cross (1965). However, the systems that he examined were polymer solutions and dispersions of solids of high viscosity that showed non-linear behaviour even at high shear rates ($1{\cdot}5 \times 10^4 sec^{-1}$). An attempt to represent the results in Fig. 2 by the equations of Sherman (1959) was not successful.

THE EFFECT OF PARTICLE SIZE ON EMULSION VISCOSITY

The precise influence of concentration cannot be assessed quantitatively unless all the emulsions have the same particle size (Sherman, 1963). A series of experiments was therefore made to ascertain the effect of variation of globule size on emulsion viscosity. The results are given in Table 2.

When the particle size was decreased, the viscosity of emulsions containing a soap concentration of 1·0% and below changed little whereas those of higher soap content showed an increased viscosity, as expected from the work of Richardson (1950), Lawrence & Rothwell (1957) and Sherman (1960).

The particle sizes (Table 1) ranged from 2 to 5 μ giving a mean particle size in the region of 3·5 μ. For this particle size the relative limiting viscosity for each laurate concentration was found from a plot of relative viscosity against mean volume diameter at each volume fraction. The change in relative viscosity with soap concentration at constant particle size (Fig. 3) shows the same maximum at 5% soap as in Fig. 2.

THE ADDITION OF ELECTROLYTES

It has been shown from the above work and by Shotton & Davis (1967) that increased soap concentration causes aggregation in the region of 1·0% with a maximum state of aggregation and aggregate size at 5·0% of soap. A possible explanation of the increase in aggregation with soap concentration is the Derjaguin-Verwey-Overbeek (D–V–O) theory (Derjaguin, 1940; Verwey & Overbeek, 1948) of colloidal stability. The thickness of the double layer and hence the forces of interparticulate repulsion are reduced as the counter ion content rises because of the increased soap concentration. The theory does not, however, provide an explanation of the aggregation maximum.

The mechanism of aggregation was investigated by the addition of various potassium salts (laurate, acetate, sulphate, chloride) to a standard liquid paraffin emulsion of 20% oil and 1·0% soap (a system beginning to show aggregation). After each addition the flow curve was measured and η_{rel}^{app} calculated. The effect of added sulphate, chloride and acetate was similar, with the relative viscosity being slightly increased. Potassium laurate shows a great increase in viscosity with increase in salt concentration (Fig. 4). The effect of adding additional laurate to a laurate emulsion, up to a given concentration, is the same as using laurate solutions of the given concentration initially.

From double layer considerations the results should be examined on the basis of added counter ion concentration. This has been done in Fig. 5

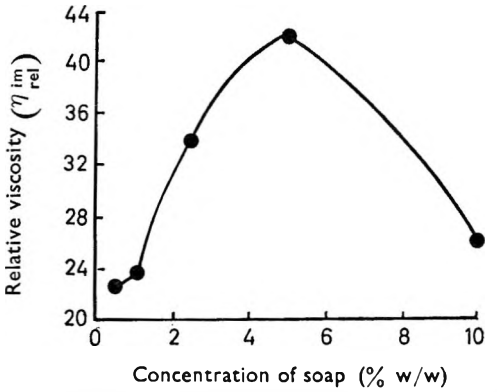

FIG. 3. The change in relative viscosity with potassium laurate concentration for liquid paraffin-potassium laurate emulsions at constant mean particle diameter (3·5 μ). Volume fraction = 0·63.

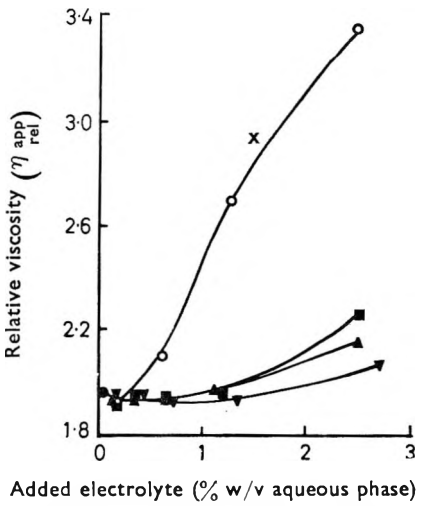

FIG. 4. The effect of added electrolyte on the relative apparent viscosity of a standard liquid paraffin-potassium laurate emulsion. Soap concentration = 1·0%. Volume fraction = 0·22. ● original system, ▲ chloride, ■ sulphate, ▼ acetate, ○ laurate, × equivalent laurate system from Table 1.

where the counter ion concentration is expressed in terms of the emulsion conductivity. The difference between laurate and the other electrolytes is further emphasized.

MICROELECTROPHORESIS

The mobility of liquid paraffin droplets increased up to a maximum at the critical micelle concentration (CMC) of the soap (0·6% w/v) and then fell slightly (Fig. 6). The zeta potential at the maximum, calculated from the Helmoltz-Smoluchowski equation, gives a value of −120 mV.

A sample of the emulsion used for the electrolyte–viscosity studies was diluted with continuous phase containing different concentrations of

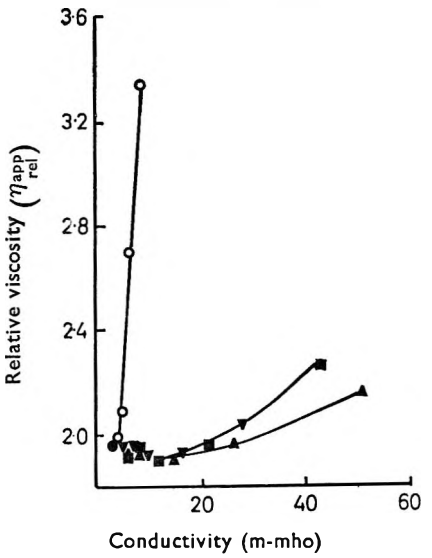

FIG. 5. The effect of counter ion concentration of the relative apparent viscosity of a standard liquid paraffin emulsion (counter ion concentration expressed in terms of conductivity). ● original emulsion, ▲ chloride, ■ sulphate, ▼ acetate, ○ laurate.

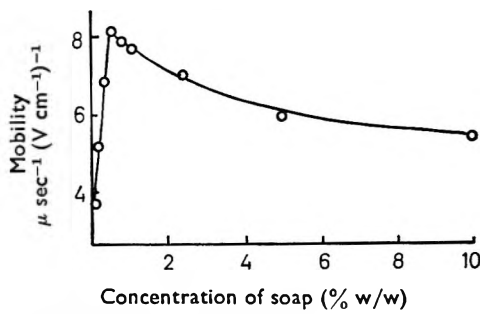

FIG. 6. The mobility of dispersed liquid paraffin in potassium laurate solutions of different concentrations.

chloride and laurate. The results (Fig. 7) show that chloride produces the expected reduction in zeta potential (D–V–O theory) whilst laurate has little effect.

Discussion

THE NATURE OF THE AGGREGATION

Two theories have been advanced to explain the increase in viscosity of a particulate system on aggregation. Goodeve (1939) and others considered link formation between particles and the work done in breaking them, whilst Mooney (1946) dealt with the entrapping of continuous phase and the subsequent increase in volume fraction. In previous work (Shotton & Davis, 1967), the volume of entrapped continuous phase

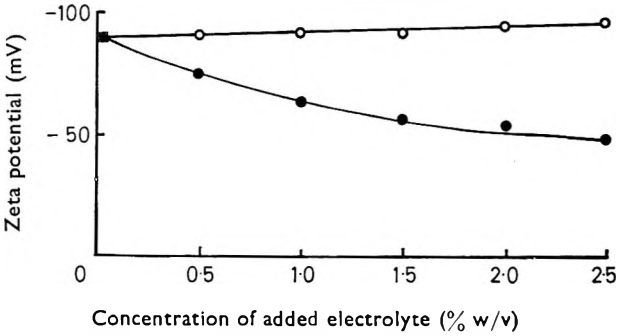

FIG. 7. The change of zeta potential of a liquid paraffin-potassium laurate emulsion in 1·0% potassium laurate solution containing added electrolyte. ○ laurate, ● chloride.

increased with soap concentration at all concentrations and therefore does not provide an explanation of the viscosity maximum. An estimate of interparticulate forces, obtained from the α value of the Cross (1965) equation, gives a maximum at 5·0% soap concentration. We conclude that the shape of the viscosity-concentration curve is due to particle-particle interaction (link formation) of the Goodeve type. Although continuous phase will be entrapped in the aggregates the contribution to viscosity appears to be small.

Many workers have explained the aggregation of particulate systems by the D–V–O theory of colloidal stability with aggregation caused by a reduction in the zeta potential. The present results are contrary to this theory, for at 1·0%, where aggregation commences, the zeta potential is only slightly lower than the maximum at the CMC, and above a 5·0% soap concentration disaggregation occurs. The electrolyte addition experiments show that the action of laurate is different from that of strong electrolytes. The latter give the classical counter ion effect of increased aggregation with increasing concentration, whilst laurate produces a far greater aggregative effect. It appears that laurate causes aggregation through the anion and not the cation (counter ion).

Cockbain (1952) suggested that states of disaggregation occurring sometimes between well-defined states of aggregation could be accounted for if adsorption of soap took place as a secondary layer at concentrations above the CMC. At concentrations just above the CMC single soap molecules would be adsorbed with their hydrocarbon chains orientated towards *the aqueous phase*. Such particles in an aqueous medium would be hydrophobic and aggregation would occur by hydrophobic bonding. At higher concentrations competitive adsorption of micelles would leave the outer surfaces of the particles hydrophilic and result in disaggregation. Cockbain's theory provides a far better explanation of the results of the present work than the D–V–O theory although the mechanism does appear at first sight to be rather improbable. Doubts have been expressed by Higuchi, Okada & Lemberger (1962) and by Becher (1965) who considered that the theory was improbable on stereometric grounds and put

forward his own explanation, based on the limited coalescence theory of Wiley (1954). It was considered that the "bleeding off" of surfactant molecules from the interface to the micelles, at the CMC, would lead to aggregation. It would seem, however, that too much attention has been paid to Cockbain's statement that aggregation occurs at concentrations at or a little greater than the CMC of the soap. In some cases Cockbain's results show, as do those of the present work, that a "little greater than the CMC" is almost twice the CMC.

The main assumption of Cockbain's theory is that multi-layer adsorption can occur in emulsion systems and the evidence for this will be discussed.

(*a*) The air-water interface: Although much of the work on the adsorption of surfactants at the air-water interface has indicated that using single pure agents the film is monomolecular, Adam (1941), Dixon, Judson & Salley (1954) and Ross (1945) consider multilayer formation to be possible, and Molliet, Collie & Black (1961) point out that multilayers may develop when two or more agents react to form a complex film.

(*b*) The solid-water interface: The adsorption of surfactants onto silver halide sols has been studied by Matijevic & Ottewill (1958) and Ottewill & Watambe (1958) and maxima and minima turbidities explained by multilayer formation and hydrophobic bonding. Powder suspensions were investigated by Griener & Vold (1949) and Vold & Konecny (1949) who attributed the maximal suspending power of the surfactant to a sharp drop in zeta potential. Doscher (1950), however, found and we have confirmed that the zeta potential was little affected by soap concentration, and he postulated a mechanism similar to that of Cockbain (1952). Moore & Lemberger (1963) and Somasundarin, Healy & Fuerstenau (1966) have suggested a film-film bridging mechanism for the maximum suspendibility of powders by surfactants.

The limitation of the D–V–O theory in explaining the aggregation of latex particles was demonstrated by Higuchi, Rhee & Flanagan (1965). It was considered that the surfactant (Aerosol O.T.) formed a weakly adsorbed, thick, hydrophilic layer, although they admitted that this was difficult to envisage.

(*c*) The oil-water interface: Recently, Cockbain's suggestion of multilayer formation and film-film interaction has been used by Lemberger & Mourad (1965) to explain why Aerosol O.T. was more effective than electrolyte in the aggregation of hexadecane emulsions. Riegelman (1962) considered that micellar adsorption would occur when a co-emulsifier such as a long chain alcohol was present, a similar idea to that of Molliet & others (1961) for the formation of multilayers at the air-water interface.

The evidence suggests that multilayer formation will occur if a mixed emulsifier film is present. Davis & Bartell (1941) and Martin & Hermann (1941) have concluded that the stability of soap emulsions is due to the formation of acid soap by hydrolysis at the interface, and resulting in a complex film of the Schulman & Cockbain (1940) type. Interfacial

hydrolysis will in fact occur even at high pH where the bulk hydrolysis is negligible (Eagland & Franks, 1960).

It may be postulated that in emulsions stabilized by emulsifiers that can be hydrolysed at the interface to produce a complex film, multilayer formation will take place at certain emulsifier concentrations, with the resultant formation of interparticulate bonds of the Cockbain type.

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The use of supports in the lyophilization of oil-in-water emulsions

M. LLADSER, C. MEDRANO AND A. ARANCIBIA

The results of drying oil-in-water emulsions by lyophilization have shown this process could be applied to emulsions having dissolved in the aqueous phase a solid material which acts as support for the oily phase after the water has been removed. The rate of creaming and the globule size distribution were also investigated. The tests were made before and immediately after lyophilization, and after 20 and 40 days of storage at room temperature (18–20°) at 0° ±1° and at 40° ±1°. The emulsions were coarser and creaming rate was increased after lyophilization.

THE preparation and the stability of emulsions are problems of primary interest (Becher, 1965; Garrett, 1965). The instability of emulsions is mainly characterized by creaming and coalescence, processes arising especially during ageing and storage. In 1960, Richter & Steiger-Trippi examined the drying of emulsions by spray-drying techniques. Drying emulsions so that they can be reconstituted when required may solve some of the problems of ageing and storage. We report a new approach to the drying of emulsions. Some of the properties of the resultant product have also been investigated.

Experimental

PREPARATION OF EMULSIONS

The internal phase was liquid petrolatum (U.S.P. XVII) and the external phase recently distilled water. Sorbic acid was the preservative (50 mg % w/w).

Mixtures of Tweens and Spans were used as emulsifying agents. Previous experiments with these agents showed that a mixture of polysorbate 80 (HLB 15) and sorbitan mono-oleate (HLB 4·3) 62·6 and 37·4% respectively was suitable. This mixture was used at 20% of the oil phase.

USE OF SUPPORTS

Two types of supports were used:

(*a*) *Crystalline.* D-(−)-mannitol (BP 1958), urea (Hopkin and Williams Ltd.), glycine (Riedel), sorbitol (Hopkin & W. Ltd.), glucose (pharmaceutical grade), sucrose (pharmaceutical grade), lactose (Merck Sharp & Dohme).

(*b*) *Colloidal.* Sodium alginate (B.D.H.), polyvinylpyrrolidone, bentonite, acacia, aerosil (Pharmaceutical grade), hydroxyethylcellulose (Cellosize WP-4400 Union Carbamide & Chemical Co.).

The crystalline supports and polyvinylpyrrolidone were used at concentrations of 13·3%; the colloidal type at concentrations of 1 or 2%, except the acacia which was used at 5%. The supports were used singly or in admixture. When the support was of the gel type, it was allowed to swell in water for 12 hr.

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SUPPORTS IN THE LYOPHILIZATION OF OIL-IN-WATER EMULSIONS

METHOD OF PREPARATION OF EMULSIONS

Both phases of the emulsion were heated separately in a water bath at 70° and premixed with a stirrer at approximately 4,000 rev/min for 5 min. The resulting coarse emulsion was then passed seven times at high pressure through an ultrasonic homogenizer (Minisonic Four Homogenizer "Ultrasonics Ltd.").

Basic formula of the emulsion. Liquid petrolatum, 10%; polysorbate, 1·25%; sorbitan mono-oleate, 0·75%; sorbic acid, 0·05%; support, q.s.; distilled water to make 100 g.

LYOPHILIZATION

Glass equipment was used for the lyophilization (Lyophilizer and Vapour Trap Quickfit, catalogue No. M.F.-45). A high vacuum pump was attached to this equipment. The lyophilization was effected in 500 ml flasks with 80 g of emulsion in each.

The freezing was done by the "shell-freezing" procedure (Calcagno, 1962; Rey, 1960), rotating the flasks at constant speed during 20 min in a freezing bath containing solid carbon dioxide and ethanol at −70°.

Primary drying was done during periods of 8 to 14 hr, using the freezing mixture previously described in the condenser. Secondary drying was effected in a dryer with phosphorous pentoxide at an approximate vacuum of 0·02 mm Hg for 5 days, changing the phosphorous pentoxide, if necessary, every 24 hr.

After the secondary drying, the lyophilized powder was placed in tightly closed and sealed containers of approximately 500 ml. A current of carbon dioxide was passed into the containers before the sealing. They were stored at room temperature, at 0° ±1° and at 40° ±1°.

CONTROLS

The following controls were used:

Moisture. This was determined immediately after lyophilization by drying 2 or 3 g at 105° in an oven for 24 hr.

The following tests were made on the product immediately after lyophilization and again after 20 and 40 days of storage at three different temperatures.

Reconstitution. This test was according to Lachman & Chavkin (1957). To a known amount of powder the corresponding amount of water was added, after 1 min the mixture was shaken for 15 sec. The shaking was alternated with 15 sec periods of rest. If the product assumed the nature of the original emulsion within the first 15 sec of shaking it was considered to be instantaneously reconstituted (I); if 2 to 4 periods of shaking were needed to achieve this state, it was considered good (G); if the emulsion was obtained after 4 periods of shaking, it was considered fair (F) and if the product did not take on the characteristics of the original emulsion, it was considered poor (P).

Creaming rate. To do the test, enough emulsion was placed in tubes 40 cm long to form a liquid column of 30 cm. Three drops of a 1%

solution of amaranth were added to give a better visualization of the process (Appino, Christian & Banker, 1962). The creaming was determined by measuring the height of the separate layer in the upper part of the tubes. It was expressed in cm/24 hr (Peck, De Kay & Banker, 1960). If a separation of a clear and transparent layer in the lower part of the tubes was observed, the creaming rate was related to measurement of the clear layer (Richter & Steiger-Trippi, 1960).

Method of globule measurements. A microscope with built-in light source (Laborlux Ernst Leitz GmBH Wetzlar), a micrometer (Leitz Wetzlar 12·5×), a cavity slide and an immersion lens were used.

A dilution 1/200 with an aqueous solution of propylene glycol at 75% (v/v) was used (Levius & Drommond, 1953; Mullins & Becker, 1956; Peck, DeKay & Banker, 1960), using a micro-calibrated pipette. In each determination, the diameter of 500–1,000 globules was measured. Ten fields were read on each sample, following the order proposed by Richter and Steiger-Trippi. But according to Münzel, Büchi & Schultz (1959) instead of counting 3 globules for each position, all those globules that fell on the scale of the micrometer and its surroundings were counted.

TABLE 1. CHARACTERISTICS OF THE EMULSIONS

Emulsion No.	Petrolatum (%)	Supports	Moisture %	RC	Mean globule diameter (μ) ± s.e.							
					Original emulsion	Reconstituted emulsion	20 days storage			40 days storage		
							R.T.	40° C	0° C	R.T.	40° C	0°C
1	10	Mannitol	0·20	I	1·66 ±0·012	2·47 ±0·026	2·76 ±0·035	2·46 ±0·027	3·10 ±0·028	2·37 ±0·022	2·26 ±0·022	2·35 ±0·017
2	10	Glycine	0·30	I	1·61 ±0·011	2·44 ±0·030	2·08 ±0·028	2·24 ±0·038	2·12 ±0·032	2·56 ±0·047	2·43 ±0·034	2·10 ±0·025
3	10	Urea	0·21	G	1·99 ±0·016	2·04 ±0·027	2·59 ±0·046	*	2·29 ±0·037	3·09 ±0·063	*	2·45 ±0·032
4	10	H.E.C. Mannitol	0·52	F	2·02 ±0·025	2·22 ±0·023	2·71 ±0·039	2·54 ±0·034	2·34 ±0·025	2·69 ±0·033	2·58 ±0·034	2·40 ±0·031
5	10	H.E.C. Glycine	0·57	F	2·27 ±0·040	1·99 ±0·020	2·42 ±0·028	2·90 ±0·032	2·29 ±0·023	2·33 ±0·021	2·79 ±0·050	2·39 ±0·024
6	10	H.E.C. Urea	0·41	G	2·00 ±0·029	2·43 ±0·036	2·57 ±0·035	2·25 ±0·029	2·32 ±0·025	2·45 ±0·035	2·37 ±0·025	2·27 ±0·025
7	10	H.E.C. Aerosil	0·30	P	1·86 ±0·016	2·41 ±0·031	2·65 ±0·040	2·60 ±0·038	2·10 ±0·027	2·72 ±0·040	2·69 ±0·040	2·34 ±0·030
8	10	Sodium Alginate	0·41	P	2·19 ±0·019	2·37 ±0·024	2·26 ±0·025	2·34 ±0·031	2·14 ±0·025	2·44 ±0·032	2·43 ±0·031	2·40 ±0·026
9	10	Aerosil Mannitol	5·30	G	2·28 ±0·047	2·31 ±0·037	2·12 ±0·033	2·30 ±0·037	1·76 ±0·024	2·17 ±0·022	2·22 ±0·031	1·95 ±0·019
10	10	H.E.C. Aerosil Mannitol	2·95	F	2·34 ±0·031	2·46 ±0·035	2·41 ±0·034	2·64 ±0·043	2·23 ±0·025	2·60 ±0·035	2·51 ±0·033	2·34 ±0·019
11	15	Aerosil Mannitol	3·40	G	2·56 ±0·050	3·24 ±0·070	2·54 ±0·054	2·86 ±0·061	2·33 ±0·044	2·62 ±0·039	2·84 ±0·052	2·39 ±0·031
12	20	Aerosil Mannitol	4·87	P	2·19 ±0·051	3·25 ±0·085	3·15 ±0·066	2·69 ±0·049	2·39 ±0·051	2·95 ±0·061	3·85 ±0·088	2·74 ±0·041

R.C. = Reconstitution characteristics. I = Instantaneous. G = Good. F = Fair. P = Poor.
* Melted at storage conditions.
H.E.C. = Hydroxyethylcellulose.
R.T. = Room temperature (18–20° C).

The globules were counted after leaving the preparation resting for 15 to 30 min to obtain stabilization (Levius & Drommond, 1953).

Results and discussion

Table 1 summarizes the results of the lyophilization. Dry products were obtained when the emulsions contained a substance which acted as support.

A series of emulsions containing a mixture of aerosil (2%) and mannitol (13·3%) as supports were examined. The emulsions contained 5, 10, 15, 20 and 25% of liquid petrolatum. With the 5 and 10% concentrations of oil in the emulsion a dry and powderable product was obtained. In emulsions containing 15% of oil, the product was unctuous, and it was even more so when petrolatum was used. Emulsions with 25% of oil phase broke during the lyophilization. Emulsions with a high proportion of oil phase seemed to need either more support or the use of another type.

Results with some supports like mannitol, glycine and urea are summarized in Table 1. Lyophilization failed with the other crystalline supports, the emulsions breaking during the process.

Colloidal materials were used to try to solve some of the negative results obtained with crystalline supports. When sodium alginate was used, a dry, tasteless and yellowish product which could be slowly redissolved in water was obtained. With other colloidal supports, the emulsions broke during the process.

Mixtures of colloidal and crystalline supports were also studied. The results are shown in Table 1.

Creaming rate. The emulsions containing crystalline supports showed a net creaming layer in the upper part of the testing tube. Lyophilization produces an increase in the creaming rate. The behaviour of emulsion 2 is shown in Fig. 1.

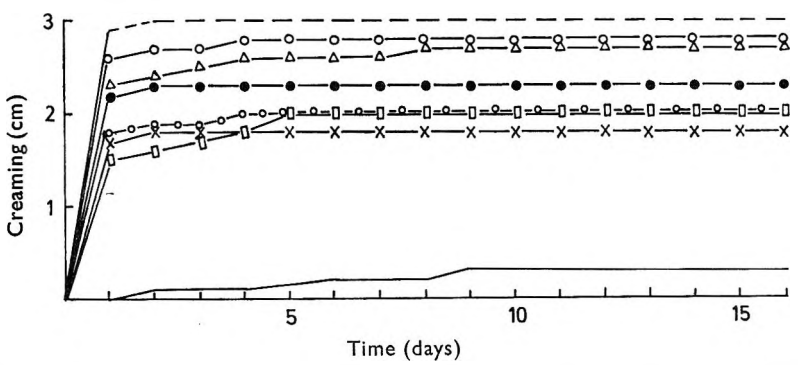

FIG. 1 Effect of time on creaming for emulsion 2 under several conditions of storage. (———) Original emulsion. (– – –) Reconstituted immediately after lyophilization. (–○–○–○–) Reconstituted after 20 days storage at room temperature. (–×–) Reconstituted after 40 days storage at room temperature. (–●–) Reconstituted after 20 days storage at 40°C. (–△–) Reconstituted after 20 days storage at 0°C. (–○–) Reconstituted after 40 days storage at 40°C. (–□–) Reconstituted after 40 days storage at 0°C.

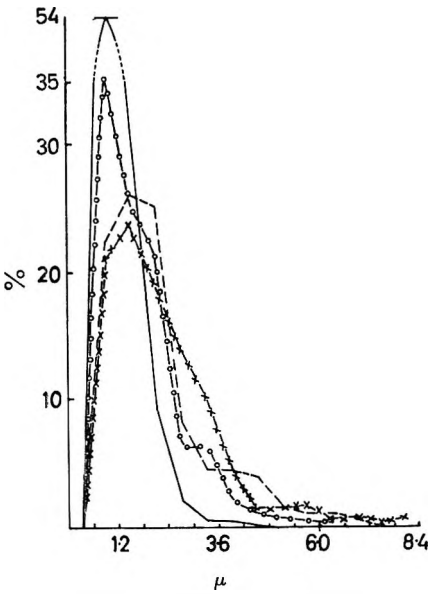

FIG. 2. Globule size distribution of emulsion 2 under several conditions of storage. (———) Original emulsion. (– – –) Reconstituted immediately after lyophilization. (–○–○–○–) Reconstituted after 20 days storage at room temperature. (–×–×–) Reconstituted after 40 days storage at room temperature.

The emulsion containing colloidal supports presented a clear layer at the bottom of the tube, in accordance with the results reported by Richter & Steiger-Trippi (1960), and the lyophilized emulsions showed a higher creaming layer than the unlyophilized preparations. A similar behaviour was observed when the emulsion contained both crystalline and colloidal supports.

It was observed that storage produced changes in the consistency and the colour of the lyophilized product. These effects were enhanced at high temperatures. For example, emulsions stored at 40° and containing hydroxyethyl cellulose greatly increased in consistency. When the support was urea, the product melted. All the emulsions stored at 40° became yellowish with time. These effects may indicate that the lyophilized products should be stored at low temperatures.

PARTICLE SIZE DISTRIBUTION

Table 1 shows the mean diameter of the particles of each emulsion and Fig. 2 shows the particle size distribution of emulsion 2. Both sets of data reveal that lyophilization produced a net increase in the mean diameter of the particle along with a displacement to higher particle size of the distribution curve.

Statistical studies (Bancroft, 1961) using Student's *t*-test showed significant differences in particle size of the original emulsions and those immediately reconstituted after lyophilization except for emulsions 3 and 9.

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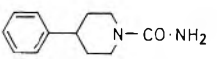
Pharmacological studies of a new antitussive, 4-phenyl-l-piperidinecarboxamide (AH 1932)

R. T. BRITTAIN, EFFIE J. LEES, P. S. J. SPENCER*

4-Phenyl-1-piperidinecarboxamide (AH 1932) markedly inhibits coughing induced in laboratory animals by chemical or mechanical irritation of the respiratory tract or by electrical stimulation of the superior laryngeal nerve. The compound is effective administered orally or parenterally and is at least as active as codeine. In contrast to codeine the antitussive activity of AH 1932 persists for 6 hr after oral administration. The evidence suggests a central site of action for AH 1932. The drug has a low acute toxicity in mice and rats, is devoid of analgesic activity, has no effect on the respiratory system and does not affect gastrointestinal propulsion. Cardiovascular effects are minimal. AH 1932 possesses weak spinal interneuron blocking activity unlikely to limit its use as a cough suppressant.

ADEQUATE doses of narcotic antitussive agents are effective in supressing cough but their use is accompanied by undesirable side-effects such as tolerance, addiction, respiratory depression, nausea and constipation. These limitations emphasize the need for new effective agents having selective antitussive properties. In animal tests 4-phenyl-l-piperidinecarboxamide (AH 1932) appears to be such a compound.



AH 1932

Experimental

ACUTE TOXICITY

Acute toxicity was determined following oral administration in male albino mice, Glaxo A_2G strain, weighing 18–22 g and male albino Wistar rats weighing 110–130 g. Deaths were recorded at 7 days. LD 50 values were calculated by the method of Litchfield & Wilcoxon (1949).

EFFECTS ON NORMAL BEHAVIOUR IN THE MOUSE, RAT, RABBIT, CAT AND DOG

The test compound was administered orally at different dose levels to each species as shown in Table 1 and the onset, character and intensity of drug effects were observed. In the mouse and rat, in addition to visual assessment of drug effects, the animals were handled to obtain information about their muscle tone, coordination and reflexes by a method similar to that described by Irwin (1963). The animals were then left undisturbed to recover from the effects of these manipulations. The process was repeated at intervals to assess the duration of drug activity and the time at which peak effects occurred. In the rabbit, cat and dog only gross changes in behaviour following drug administration were recorded.

ANTITUSSIVE ACTIVITY

Antitussive activity was determined against cough induced by chemical or mechanical irritation of the respiratory tract in conscious guinea-pigs or anaesthetized cats respectively and also by electrical stimulation of the superior laryngeal nerve in anaesthetized cats.

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TABLE 1. SPECIES, STRAIN, SEX, WEIGHT RANGES AND NUMBER OF ANIMALS USED IN BEHAVIOURAL STUDIES

Species	Strain	Sex	Body weight range	No. animals per dose level
Albino mouse	A_2G (Glaxo)	Male	18–22 g	3
Albino rat	Wistar	Male	110–130 g	3
Rabbit	Dutch	Male & Female	1·8–2·6 kg	1 Male, 1 Female
Cat	—	Male & Female	2·0–4·2 kg	1 Male, 1 Female
Dog	Mongrel	Male & Female	8·0–13 kg	1 Male, 1 Female

Guinea-pig. Experimental coughing was induced by inhalation of ammonia vapour (Winter & Flataker, 1954). Male albino guinea-pigs weighing 250–450 g were placed individually in a Perspex chamber through which air was passed either directly or after bubbling through 20% aqueous ammonia. A rotameter was included in the system to ensure constant air flow and an air escape prevented build up of pressure. A recording tambour was joined to the chamber to record changes in pressure due to coughing. Guinea-pigs were exposed to ammonia vapour for 90 sec and after this time air was passed directly into the chamber for a further 90 sec. The number of coughs during the 3 min period was determined. [The record of each cough is characteristic, is easily distinguishable from responses produced by sneezing, deep expirations, locomotor or grooming movements.] On the following day the procedure was repeated after animals had received test or reference compounds orally 90 min before exposure to the amonia vapour. The chamber was well ventilated between individual tests and the ammonia solution was changed after 6 experimental test periods. Five or 6 animals were used in each group. Antitussive activity was expressed as the percentage inhibition of coughing, each animal serving as its own control. While a high variation existed between animals in their sensitivity to ammonia, the responses of individual guinea-pigs did not vary by more than 5% on repeated exposure to ammonia. ED 50 values were calculated from the regression line of percentage inhibition plotted against dose.

Antitussive activity was also determined in guinea-pigs against coughing induced by sulphur dioxide. The method used was essentially that described by Miller, Robbins & Meyers (1963).

Cat. Male and female cats weighing 2·8–4·5 kg were anaesthetized with pentobarbitone sodium, 45 mg/kg intraperitoneally. Coughing was induced either mechanically by passing a polythene tube down the trachea until it touched the carina and then withdrawing it immediately (May & Widdicombe, 1954), or by electrical stimulation of the superior laryngeal nerve (Domenjoz, 1952). In the latter method the central end of the ligated nerve was stimulated for 5–15 sec with a train of rectangular pulses of 1–10V strength and 10–15 msec width, at a frequency of 5–10 impulses/sec. The interval between successive stimuli was 5 min in both techniques Diaphragmatic movements were recorded on smoked paper by an isotonic lever attached by a thread to the skin over the xiphisternum. Inhibition of the coughing was assessed by measuring the height of the records obtained, and expressed as a percentage of the control height.

ANALGESIC ACTIVITY

Groups of 10 mice received the test compound orally. One hr after drug administration, analgesic activity was investigated by determining the ability of a compound to inhibit writhing induced by phenylquinone (Brittain, Lehrer & Spencer, 1963) and to inhibit nociceptive response in a standardized tail-pinch method (Bianchi & Franceschini, 1954).

ANTICONVULSANT ACTIVITY

Anti-leptazol test. Groups of 10 mice received the test compound orally 90 min before a subcutaneous injection of leptazol, 100 mg/kg. The number of mice protected against the convulsive and lethal effects of leptazol was recorded.

Anti-maximal electroshock test. The test compound was administered orally to groups of 10 mice. Electric shock was applied through ear electrodes 2 hr after drug administration (Cashin & Jackson, 1962), and the number of animals showing no tonic extension of the hind limbs was recorded.

NEUROLEPTIC ACTIVITY

The method was based on the antagonism of amphetamine-induced toxicity in mice housed under crowded conditions (Burn & Hobbs, 1958). Groups of 8 mice received the test or a reference compound orally. Two hr after drug administration all mice were injected subcutaneously with amphetamine 15 mg/kg and then housed under crowded conditions as described by D'Arcy & Spurling (1961). After 4 hr the number of animals still alive in each group was recorded.

ACTION ON SPINAL REFLEXES

Male or female rabbits weighing 1·8–2·4 kg were anaesthetized with urethane 1·25 g/kg intravenously. In some experiments the knee jerk (monosynaptic reflex) was elicited at 10–30 sec intervals by tapping the patellar tendon (Palmer automatic knee jerk hammer). The method was similar to that described by Schweitzer & Wright (1938). In other experiments, flexor contractions of the tibialis anterior muscle (multi synaptic reflex) were elicited at 10–30 sec intervals by stimulation of the central end of the ligated ipsilateral superficial peroneal nerve with a train of rectangular pulses of 0·3–8 V strength and 0·1–1·0 msec width, at a frequency of 20–100 impulses per sec for a duration of 0·1–0·5 sec. Reflex contractions were recorded kymographically. Drugs were administered through a cannula in an external jugular vein.

ACTION ON CARDIOVASCULAR AND RESPIRATORY SYSTEMS

Effects in anaesthetized cats. Male or female cats weighing 2·8–4·2 kg were anaesthetized with chloralose 70 mg/kg intravenously after induction with 3% halothane in nitrous oxide and oxygen (3 : 1). Blood pressure was recorded from a femoral vein using a mercury manometer. Compounds were injected intravenously. Their effects on blood pressure or on the responses of the blood pressure to various vasoactive agents and occlusion

of the common carotid arteries, or both, were investigated. Respiratory rate and depth were also recorded in the anaesthetized cat using a Rubens non-rebreathing valve connected to a volume displacement recorder.

Effects in conscious renal hypertensive dogs. Male Beagle dogs weighing 12–15 kg were used which had been made hypertensive by application of rubber capsules to both kidneys 2–4 months before the experiment. Blood pressures were measured indirectly by application of a cuff to a carotid loop which had been exposed before the kidney operation. Blood pressure and heart rate were measured at half-hourly and then hourly intervals after oral administration of the test compound.

EFFECTS ON GASTROINTESTINAL TRACT

Compounds were investigated for their ability to inhibit gastrointestinal propulsion of a charcoal meal in mice (Brittain & Collier, 1958). Groups of 10 mice received the test compound orally. The charcoal meal was given 1 hr after drug administration and the mice killed 20 min later. The length of small intestine traversed by the meal was measured and expressed as a percentage of the total length of the small intestine.

DRUGS AND SOLUTIONS

4-Phenyl-1-piperidine carboxamide (AH 1932) is a white, odourless, tasteless, crystalline solid. It is sparingly soluble in water and was administered orally as a suspension in 5% gum acacia in water. Solutions for intravenous injection were prepared either in dilute acetic acid or in dimethylacetamide. Doses of drugs given in the text refer to the free base.

Results

ACUTE TOXICITY IN THE MOUSE AND RAT

The acute toxicities of AH 1932 and codeine following oral administration in the mouse and rat are respectively (LD 50 mg/kg with 95% fiducial limits mg/kg) AH 1932, 1,055 (887–1,255); codeine, 255 (199–306): AH 1932, 790 (637–980); codeine, 335 (293–382). Thus in these species codeine is about 2–3 times as toxic as AH 1932.

EFFECTS ON NORMAL BEHAVIOUR IN THE MOUSE, RAT, RABBIT, CAT AND DOG

Mouse. AH 1932, 50 mg/kg orally, had no visible effect on normal behaviour but after 100 mg/kg the righting reflex of animals was slightly impaired. Body posture, responses to a noxious stimulus, grooming and reactivity to a changed environment were all depressed by a dose of 200 mg/kg and these effects were more marked after an increase to 300 mg/kg. At the latter dose level there were also signs of stimulation such as restlessness, exophthalmous and mydriasis. Ptosis did not occur at any of the dose levels tested. The depressant effects of AH 1932 appeared to be due primarily to impaired muscular control.

Rat. No significant effects were observed following 100 mg/kg of AH 1932. After 200 mg/kg the animals were slightly restless and had raised body postures. Spontaneous locomotor activity was also increased but alertness was reduced. Similar but more pronounced effects were

seen after 400 mg/kg. At 800 mg/kg, the rats were very restless initially and had markedly elevated body postures but 25 min after drug administration severe ataxia developed rapidly and the animals became progressively more depressed with complete loss of the righting reflex and limb and body tone. However, in this state, corneal and pinnal reflexes were still present but ipsilateral spinal reflexes abolished.

Rabbit. The main effects following oral doses of 100 and 200 mg/kg AH 1932, were lowered body posture and reduced spontaneous motor activity. Slight catalepsy occurred in one animal which had received 200 mg/kg. A dose of 400 mg/kg also reduced respiratory rate. Following 800 and 1,200 mg/kg the righting reflex was almost abolished and animals were cataleptic; the corneal and pinnal reflexes were depressed but not absent. The behaviour of all animals was normal 24 hr after drug administration.

Cat. AH 1932 was more toxic in the cat than in other species investigated. Slight hind limb inco-ordination occurred after 100 mg/kg. After 200 mg/kg, ataxia developed in one animal and in the other there was marked catalepsy, spasticity and loss of the righting reflex. Doses of 400 mg/kg and 800 mg/kg abolished the righting reflex and marked spasticity and limb tremor developed. One of two animals receiving 400 mg/kg died and both animals receiving 800 mg/kg died.

Dog. No effects were observed after oral doses of 75 and 150 mg/kg. After 300 mg/kg vomiting occurred with restlessness, impairment of the righting reflex, tremor and limb incoordination. After 600 mg/kg restlessness was marked and the dogs were ataxic. Frequent hind limb collapse occurred in one animal but the righting reflex was not abolished. All animals were normal after 24 hr.

ANTITUSSIVE ACTIVITY

Conscious guinea-pig. The effects of orally administered AH 1932, codeine and morphine on coughing induced by ammonia vapour in conscious guinea-pigs are respectively (oral ED 50 mg/kg with 95% fiducial limits mg/kg): 36·8 (30·1–45·0); 42·1 (38·1–46·5); 16·9 (14·9–19·2).

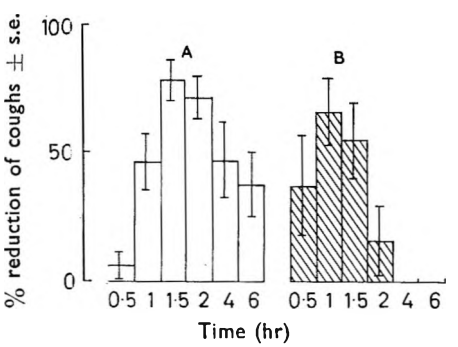

FIG. 1. The intensities and durations of the effects of AH 1932 (A) and codeine (B) on coughing induced by ammonia in conscious guinea-pigs. The ordinate gives the interval between administration of drug and exposure to ammonia vapour. The drugs were administered orally at 50 mg/kg.

Although the antitussive potency of AH 1932 was similar to that of codeine, the time courses of action of the drugs were different (Fig. 1). The onset of antitussive activity of AH 1932 was delayed but its duration of action was much longer than that of codeine. In a further experiment on conscious guinea-pigs the antitussive activity of AH 1932 and morphine were compared against coughing induced by sulphur dioxide. In this test the drugs were administered orally and AH 1932 and morphine were found to be equipotent.

Anaesthetized cat. The effects of AH 1932 and codeine in inhibiting coughing induced by mechanical stimulation of the trachea or electrical stimulation of the superior laryngeal nerve are summarized in Table 2. The record of one experiment is given in Fig. 2. In both series of experiments AH 1932 was marginally more active than codeine particularly at the lower dose levels investigated. At equipotent doses the durations of action of the drugs were similar. The effects of AH 1932 and codeine administered simultaneously to anaesthetized cats with mechanically induced coughing were additive.

TABLE 2. ACTIVITY OF AH 1932 AND CODEINE IN PREVENTING COUGHING INDUCED BY MECHANICAL STIMULATION OF THE TRACHEA OR ELECTRICAL STIMULATION OF THE SUPERIOR LARYNGEAL NERVE IN THE ANAESTHETIZED CAT

Compound	Dose mg/kg i.v.	Percentage inhibition (± s.e.) of coughing induced by	
		Mechanical stim.	Electrical stim.
AH 1932	0·125	12·5	—
	0·25	37·7 ± 3·0 (4)	36·9 ± 4·5 (4)
	0·5	64·4 ± 7·4 (4)	62·4 ± 4·7 (5)
	1·0	74·8 ± 5·7 (5)	78·9 ± 6·6 (5)
Codeine	0·25	21·3 ± 9·6 (3)	22·0
	0·5	45·3 ± 5·8 (5)	37·8 ± 9·6 (4)
	1·0	74·4 ± 5·1 (5)	77·1 ± 3·4 (5)

Figures in parentheses indicate number of determinations

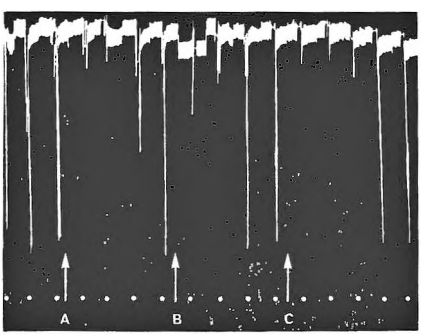

FIG. 2. Cat, 2·5 kg, pentobarbitone anaesthesia. Movements of the xiphisternum in response to stimulation of the superior laryngeal nerve (dot) at 5 min intervals. At A and B, AH 1932 injected intravenously at 1·0 and 0·5 mg/kg respectively. At C, codeine 1·0 mg/kg intravenously.

ANALGESIC ACTIVITY

The respective results of phenylquinone and tail-pinch tests in mice after oral administration of AH 1932, codeine or morphine are (ED 50 mg/kg with fiducial limits mg/kg): AH 1932 $>$ 150, $>$ 150; codeine, 12·0 (5·7–25·2); 27·6 (16·8–45·9); morphine, 3·2 (2·2–4·7); 12·8 (7·9–20·7). AH 1932 is not an effective analgesic in these tests, though codeine and morphine were highly active as expected.

ANTICONVULSANT ACTIVITY

AH 1932, 150 mg/kg orally, failed to protect animals against convulsions and deaths caused by leptazol. However, the compound possessed weak anti-convulsant activity against seizures induced by maximal electric shock and was about 1/30 as active as phenytoin.

NEUROLEPTIC ACTIVITY

AH 1932, 150 mg/kg orally, was inactive in the anti-amphetamine test. In contrast, the ED 50 for chlorpromazine in this test was 2·74 (1·55–4·85) mg/kg. It can be concluded therefore that AH 1932 has no neuroleptic activity.

ACTION ON SPINAL REFLEXES

AH 1932, 5 mg/kg intravenously, depressed contractions of a tibialis muscle elicited through a multisynaptic pathway (flexor reflex) by about 50% for 7–10 min. The depressant effect was more marked after 10 mg/kg. At these dose levels AH 1932 had little or no effect on contractions of the quadriceps femoris muscle elicited through a monosynaptic pathway (patellar reflex). These results indicate that AH 1932 possesses spinal interneuron blocking activity.

ACTION ON CARDIOVASCULAR AND RESPIRATORY SYSTEMS

Effects in the anaesthetized cat. Intravenous doses of 1–5 mg/kg AH 1932 had no significant effect on arterial blood pressure or on blood pressure changes due to vasopressor agents such as noradrenaline, tyramine nicotine and angiotensin. A higher dose, 10 mg/kg, caused variable but transient effects on the blood pressure but these effects could also be reproduced by injection of the solvent alone. This dose level also partially depressed for a short period the pressor responses to occlusion of the common carotoid arteries and to injected noradrenaline, tyramine and nicotine. AH 1932, 1–10 mg/kg, had no significant effect on heart rate and the ECG.

AH 1932 produced no marked effect on respiration. Intravenous doses of 2·5–10 mg/kg produced initial stimulation of respiratory rate and depth but again this response could usually be attributed to the solvent used. No depression of respiration was seen with any of the dose levels investigated.

Effects in conscious renal hypertensive dogs. Three hypertensive dogs received AH 1932, 50 mg/kg orally, for 3 consecutive days. The compound had no significant effect on blood pressure or heart rate nor did it cause noticeable changes in the behaviour of the animals.

EFFECTS ON GASTROINTESTINAL TRACT

The comparative effects of AH 1932 and codeine after oral administration in the mouse on gastrointestinal propulsion of a charcoal meal are illustrated in Fig. 3. In contrast to codeine, AH 1932 at high oral doses did not inhibit transport of the charcoal meal along the gastrointestinal tract.

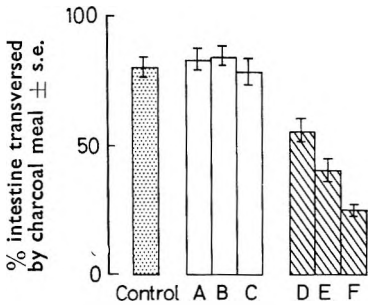

FIG. 3. The effects of AH 1932 and codeine on the gastrointestinal propulsion of a charcoal meal in conscious mice. At A, B and C, AH 1932 administered orally at 25, 50 and 100 mg/kg respectively. At D, E and F, codeine administered orally at 25, 50 and 100 mg/kg respectively. All drugs given 1 hr before, and animals killed 20 min after, the administration of the test meal.

Discussion

4-Phenyl-1-piperidinecarboxamide (AH 1932) consistently showed marked activity in preventing coughing induced in laboratory animals by chemical or mechanical irritation of the respiratory tract or by electrical stimulation of the superior laryngeal nerve. In these tests AH 1932 was as active as codeine. In guinea-pigs the action of AH 1932 was slower in onset but more prolonged than that of codeine, significant antitussive activity persisting for 6 hr after oral administration. With codeine, activity usually ceased within 2 hr. The site of action of AH 1932 is not definitely known, but it is unlikely that a peripheral inhibitory action on sensory receptors is involved since AH 1932 was equally effective in blocking coughing induced by stimulation of the central end of the superior laryngeal nerve and coughing induced by irritation of the respiratory tract. These observations suggest a central site of action.

In contrast to the morphine group of antitussive agents, AH 1932 was devoid of analgesic activity as shown by its lack of activity in the phenylquinone and tail-pinch tests in the mouse. The phenylquinone test was introduced for detecting non-narcotic analgesic activity of new compounds (Siegmund, Cadmus & Lu, 1957). However, many other types of compound, for example, narcotic analgesics, parasympathomimetics, sympathomimetics, anti-inflammatory agents and monoamineoxidase inhibitors, inhibit writhing induced by phenylquinone (Hendershot & Forsaith, 1959; Brittain & others, 1963). Since AH 1932 was inactive in the phenylquinone test it may be inferred that the drug is probably also devoid of the many types of activity outlined above. The lack of analgesic activity in

AH 1932 coupled with its obvious chemical dissimilarity to morphine would also indicate that problems of tolerance and addictive liability are unlikely to occur with this compound.

Behavioural studies with AH 1932 indicated a curious mixture of stimulant and depressant effects, the latter predominating at higher doses. It was thought that the depressant effects were due mostly to impaired muscular control. The drug depresses the multisynaptic flexor reflex but not the monosynaptic patellar reflex, showing this impairment to be caused by spinal interneuron blockade. These results also rule out action of AH 1932 on the myoneural junction, on skeletal muscle and on conduction in peripheral nerves including the lower motoneurons involved in coughing. The doses necessary to cause interneuron blockade greatly exceed those which cause antitussive activity and it is therefore unlikely that the interneuron blocking effects of AH 1932 would limit its use as an antitussive agent.

AH 1932 had no effect on respiration; an advantage over morphine and related compounds, which are known to depress respiration (Krueger Eddy & Sumwalt, 1941; Wikler, 1950; Loeschcke, Sweel & others, 1953). A further advantage of AH 1932 over codeine might be its lack of action on the gastrointestinal tract, codeine being well known to cause constipation (Meyler, 1966).

The cardiovascular actions of AH 1932 are slight. At high dose levels in the anaesthetized cat there was a weak α-adrenergic blocking action. However, in conscious hypertensive dogs even higher doses given orally had no noticeable effects on the cardiovascular system. Cardiovascular effects are unlikely to limit the use of the drug as a cough suppressant.

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Protection by ethanol against the toxic effects of monofluoroethanol and monochloroethanol

D. I. PETERSON,* J. E. PETERSON, AND M. G. HARDINGE

Ethanol protects rats and monkeys against fatal doses of monofluoroethanol and monochloroethanol. In rats treated with ethanol the LD50 for fluoroethanol was found to be more than 20 times that in unprotected animals, for chloroethanol it was about 4 times greater. In monkeys too, the lethal effects of these compounds are diminished by treatment with ethanol.

ETHANOL protects experimental animals from some of the toxic effects of ethylene glycol (Peterson, Peterson & others, 1963) and animals given a lethal dose often survive if promptly treated with ethanol. Some harmful effects of ethylene glycol appear to be related to products of enzymatic oxidation that are more toxic than the parent compound. In animals protected with ethanol most of the ethylene glycol has been excreted unchanged in the urine. Oxidation of ethylene glycol is catalyzed by liver alcohol nicotinamide-adenine dinucleotide (NAD) oxido-reductase and this oxidation is competitively inhibited by ethanol (Blair & Vallee, 1966). Wacker, Haynes & others (1965) confirmed the effectiveness of ethanol treatment in man poisoned with ethylene glycol.

We have investigated the effect of ethanol on the toxicity of related compounds in rats and monkeys. We found no evidence that ethanol prevents the lethal effects of isopropyl, n-propyl, n-butyl, or n-amyl alcohols, nor is it effective in rats given diethylene glycol. Toxic effects of these compounds seem to be increased when animals are also treated with ethanol, but mortality in rats and monkeys given 2-fluoroethanol and 2-chloroethanol is sharply reduced by ethanol treatment. Experimental data supporting these latter observations are now presented.†

Experimental

The intraperitoneal LD50 values of reagent grade monofluoroethanol (Calbiochem) and reagent grade monochloroethanol (Eastman Kodak) were determined using male Sprague-Dawley rats, of 140–160 g. Ten animals were used to obtain each point on the curves. The toxicity of these substances to rats subsequently treated with ethanol (25% v/v in water) was then determined similarly.

Treated rats were given an initial dose of 2 ml/kg of ethanol intraperitoneally 15 min after receiving the halogenated ethanol. Subsequent doses of ethanol averaging 1·5 ml/kg, were given every 4 hr. The dose was sometimes varied because of variation in ethanol toleration, or the amount required for protection, in different animals. Ethanol treatment was continued for 84 hr in rats given chloroethanol and in those animals given less than 30 mg/kg of fluoroethanol. It was necessary to treat

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† A preliminary report of this work appeared in *Fedn Proc. Fedn Am. Socs exp. Biol.*, (1964), **23**, 199.

some animals receiving higher doses of fluoroethanol for up to 6 days to prevent convulsions.

Twenty squirrel monkeys (*Samiri sciurea*), 630–775 g, were divided into four groups, each of three males and two females. Monofluoroethanol was administered to two groups intraperitoneally at 100 mg/kg and chloroethanol to the other two groups at 150 mg/kg. One of the fluoroethanol- and one of the chloroethanol-treated groups was also given 1·5 ml/kg of ethanol by orogastric tube 15 min later. Subsequent doses of ethanol were given (average dose 1·0 ml/kg every 4 hr) for 96 hr. Individual monkeys, like the rats, required different doses of ethanol. Each monkey was also given 20 ml/kg of water every 4 hr. Five other monkeys of the same species, similar in weight and sex distribution to those in the groups, were given 250 mg/kg of chloroethanol intraperitoneally, followed by ethanol treatment similar to those given the smaller dose.

To evaluate the effect of barbiturate anaesthesia on fluoroethanol toxicity, four groups of 10 male Sprague-Dawley rats (each of 150 g) were used. Animals in groups 1 and 2 received 3 mg/kg and those in groups 3 and 4, 6 mg/kg of fluoroethanol intraperitoneally. Groups 1 and 3 were initially anaesthetized with 70 mg/kg of methophenobarbitone intraperitoneally and then sufficient barbiturate to keep them lightly anaesthetized until death. Survival times were recorded. The significance of difference between mean survival times was determined by Student's *t*-test.

Results

The intraperitoneal LD50 values for rats given monofluoroethanol or monochloroethanol alone are: 1·75 mg/kg (1·26–2·4)‡ for fluoroethanol and 44·0 mg/kg (40·0–48·4) for chloroethanol. With ethanol protection the LD50 values were >60·0 mg/kg and 175·0 mg/kg (139·0–220·0) respectively. Thus ethanol gave significant protection from the toxic effect of either compound, but was more effective against fluoroethanol.

All monkeys given fluoroethanol or chloroethanol but no ethanol, died. All monkeys given ethanol survived after 100 mg/kg of fluoroethanol. One monkey developed severe muscle spasm when ethanol treatment was briefly discontinued after 4 days, recovering when treatment was resumed for a further 24 hr. Monkeys given the chloroethanol, 150 mg/kg, and subsequent treatment with ethanol also survived. Those given 250 mg/kg chloroethanol appeared to be protected for 36–48 hr but they then became comatose and died despite continued treatment.

The optimum dose of ethanol varied widely. Rats given a uniform dose were often continuously in stupor or appeared to have completely recovered from the effects of ethanol 4 hr after it had been given, irrespective of the amount of fluoroethanol, though the dose ranged from 20–60 mg/kg. The ten rats given 60 mg/kg of fluoroethanol were observed closely in an attempt to find an optimum protective dose of ethanol for each rat. None died until the fifth day when treatment with ethanol was briefly discontinued. The survivors (7/10) were then successfully

‡ 95% confidence limits determined according to Litchfield & Wilcoxon (1949).

treated by giving ethanol for further 24 hr. Four of these had then lost more than one third of their starting weight.

The dose range of chloroethanol was 150–250 mg/kg. Even at a dose of 150 mg/kg 3/10 rats died and despite optimum dosage of ethanol complete protection against chloroethanol was not achieved.

Rats and monkeys dying after fluoroethanol usually had severe convulsions while those dying after chloroethanol became somnolent and later deeply comatose. Treble (1962) found that some rats given fluoroethanol died without having convulsions. Chenoweth (1949) reported that rhesus monkeys given fluoroethanol may die of ventricular fibrillation. In our experiments with fluoroethanol, all rats and monkeys not given ethanol, and that were observed continuously, did convulse. Some died immediately after the convulsion, but usually there was a postictal depression during which time the animals were stuporous.

Rats given fluoroethanol had no convulsions when lightly anaesthetized with methophenobarbitone. The period of survival was extended by this treatment (3 mg/kg, $P < 0.01$; 6 mg/kg, $P < 0.001$) but mortality was not reduced for animals receiving these dosages of fluoroethanol (Table 1).

TABLE 1. THE LENGTH OF TIME RATS SURVIVED AFTER 2-FLUOROETHANOL WAS GIVEN ALONE AND WHEN THE ANIMALS WERE KEPT ANAESTHETIZED WITH METHOPHENOBARBITONE. There were ten rats in each group.

2-Fluoroethanol dose mg/kg	Survival time (hr)	
	Mean s.d.	Range
3 with methophenobarbitone* ..	48·6 ± 23·0	(18–96)
3 without ,, ..	20·8 ± 14·8	(6–52)
6 with ,, ..	16·4 ± 15·9	(5–56)
6 without ,, ..	5·6 ± 3·7	(2– 8)

* Initial dose of 70 mg/kg intraperitoneally with subsequent dosage sufficient to maintain light anaesthesia.

Discussion

Monofluoroethanol and monochloroethanol are used in industry and in the laboratory. Monofluoroethanol has the same degree of toxicity as fluoroacetate (Williams, 1959, Chenoweth, 1949) which is one of its metabolic products. The toxic effects of fluoroethanol may depend on its enzymatic oxidation to the acetate. The oxidation of chloroethanol, a compound which has caused at least seven fatalities recorded in recent literature (Dreisbach, 1966) may follow a metabolic pathway similar to that for fluoroethanol (Williams, 1959). Both these halogenated ethanols are reported to serve as substrates for crude preparations of liver alcohol NAD oxidoreductase (Bartlett, 1952, Bernheim & Handler, 1941).

We have found ethanol treatment gives significant protection to rats or monkeys after a lethal dose of either halogenated ethanol. The LD50 for either compound was significantly raised. With fluoroethanol none of the protected rats given 60 mg/kg died before the fifth day while the mean survival period for unprotected rats given only 6 mg/kg was 5·6 hr. Rats or monkeys given a lethal dose of fluoroethanol nearly always died within 48 hr if ethanol was not given.

Treatment with ethanol was surprisingly effective in the rats given

fluoroethanol. Survival seemed to be a function of the precision of the ethanol treatment rather than the dose of fluoroethanol. Treatment was less effective in those given chloroethanol; this may be because of greater toxicity of the parent compound, from the use of another metabolic pathway or from a less active inhibition of enzymatic oxidation.

Central nervous system depression by ethanol apparently plays a very minor role in protection against fluoroethanol since light barbiturate anaesthesia prevented convulsions but caused little if any change in mortality. The fatal period was however lengthened. Hutchins, Wagner & others (1949) found this to be so in dogs given fluoroacetate and they reported that ethanol offered a minor degree of protection against fluoroacetate. This protection was thought by these authors to be due to acetate supplied by the oxidation of ethanol and was unlikely to account for the much greater degree of protection offered by ethanol against fluoroethanol and chloroethanol.

Fluoroethanol is apparently excreted slowly since rats given the larger doses required six days treatment to prevent convulsions.

Blair & Vallee (1956) showed both fluoroethanol and chloroethanol were substrates for human liver alcohol NAD oxidoreductase and that their oxidation rates relative to ethanol were 0·10 and 0·20 respectively. Protection offered by ethanol against the toxicity of mono-halogenated ethanols may be due to competitive inhibition of oxidation; the parent compound being excreted without being oxidized to more toxic metabolites.

Pattison, Howell & others (1956) reported that several ω-fluoro-alcohols with an even number of carbon atoms were more toxic than those with an odd number of carbon atoms. It has been suggested that the compounds with an even number of carbon atoms are metabolized by β-oxidation to fluoroacetate. It is possible that ethanol will also protect animals from the lethal effects of these compounds.

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Anethole and fenchone in the developing fruits of *Foeniculum vulgare* Mill.

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The anethole and fenchone content of the developing fruits of bitter and sweet varieties of *Foeniculum vulgare* Mill. (fennel) has been assayed by gas chromatography over three seasons. Anethole continuously increases in both varieties to about 22 mg/100 fruits. Fenchone is present at all stages of development of both varieties, and continuously increases to about 10 mg and 2 mg/100 fruits in the bitter and sweet varieties respectively. Greater oil yields from the bitter fruit are thus due to their higher fenchone content and lower weight.

THE principal flavouring constituents of *Foeniculum vulgare* Mill. (fennel) are the aromatic *trans*-anethole and the terpenoid (+)-fenchone, which may comprise up to 90% and 22% respectively of the essential oil of different specimens of this umbelliferous fruit (Tóth, 1967a). The product of these two dissimilar oil constituents in developing fennel fruits is compared in this communication with the situation previously observed for carvone (Betts, 1965), where a "specific level" was observed in fruits that produce it.

Experimental

Plant material. This was identified, grown, marked and sampled as previously described (Betts, 1965), samples being taken during three growing seasons at various stages of fruit development from "bitter" (var. *vulgare* (Mill.) Thelung) and "sweet" (var. *dulce*) varieties. Plants of the latter variety were raised from "seed" obtained from Italy, whilst the former variety was found growing in Myddelton House gardens, Enfield. The two varieties are distinct in appearance, the bitter variety being a taller, but less robust plant bearing smaller flowers and fruits than the sweet variety, and having a distinct camphoraceous taste and odour of fenchone overlaying the sweetness of anethole.

Extracts. Fruit collected were dropped as soon as possible into volumetric flasks (5, 10 or 20 ml according to the degree of development) containing enough absolute ethanol to cover them; the contents were then made up to volume with ethanol. After at least six months storage in the dark at room temperature (20°), the volume was again made up if necessary and aliquots (1 μl) were taken for qualitative and quantitative gas chromatography.

Gas-liquid chromatography. Pye 104 apparatus was used isothermally at 140° with a flame ionization detector at the oven temperature. A glass column (5 ft), internal diameter 4 mm was used, packed with 15% Carbowax 20M (a polyethylene glycol) on Chromosorb W (80–100 mesh), previously purged at 225°. Mobile phase nitrogen, with flow rate at column exit 40 ml/min; the hydrogen supply to the detector being at the

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same flow rate. Under these conditions the column contained approximately 2000 theoretical plates for anethole and 800 theoretical plates for fenchone.

Anethole and fenchone. These were obtained by measuring the heights of anethole and fenchone peaks from the extracts against those obtained on the same occasion from standard solutions. While the anethole standard was pure, the fenchone was of technical quality and contained an impurity of longer retention time with a peak area one-ninth that of the fenchone. Calculations of fenchone content were therefore made on the assumption that the fenchone was 90% pure. Peaks present in the extracts were identified by comparison with reference materials both on the Carbowax column and on a polyethylene glycol adipate column, and included a small peak of estragole, the allyl isomer of anethole, which was observed in all extracts. An internal standard was not used but the same operator made the injection and assays were repeated until results were consistent.

Results and discussion

Similar results were obtained for the developing fruits over three seasons, and illustrative figures are in Table 1. Results are given as

TABLE 1. OBSERVATIONS ON DEVELOPING FENNEL FRUITS 1966

Date marked i.e. in flower	Date collected	Days since marking	mg anethole in 100 fruits*	mg fenchone in 100 fruits*	% fenchone in essential oil†
		Bitter fennel			
2 Sept.	16 Sept.	14	2·9	1·6	36
2 Sept.	26 Sept.	24	5·0	2·9	37
10 Aug.	16 Sept.	37	16·6	6·6	28
10 Aug.	26 Sept.	47	18·5	6·9	27
10 Aug.	20 Oct.	71	22·4	10·0	31
		Sweet fennel			
16 Sept.	26 Sept.	10	2·5	trace	–
2 Sept.	16 Sept.	14	4·5	0·3	6
10 Aug.	16 Sept.	37	7·6	0·5	6
10 Aug.	26 Sept.	47	15·9	1·4	8
10 Aug.	20 Oct.	71	21·5	1·9	8

* Average of at least two determinations.
† Assuming the essential oil is composed only of anethole and fenchone.
Relative retention time to linalol: fenchone, 0·66; anethole 3·75.

content of substance assayed per 100 fruits (entire cremocarps) as this method is of more value in developmental studies than figures based on weight of tissue. Such values for carvone give misleading "peak" concentrations at early stages of fruit development due to the oil constituent increasing more rapidly than the dry weight (Betts, 1965). Fennel fruits were extracted by cold ethanolic maceration and not by steam distillation, for whilst the latter process rapidly completes the removal of carvone from whole fruits, the removal of anethole requires repeated co-distillations with water. Several months extraction was allowed to ensure that

the oil constituents were evenly distributed throughout the contents of the volumetric flask. That extraction was complete was shown by obtaining the same assay several months later. There was no gas chromatographic evidence of anethole decomposition, as shown by the appearance of an anisaldehyde peak for example, during storage of the extracts. Gas chromatography is preferable to thin-layer chromatography for qualitative examination of fennel as anethole is always partly decomposed to anisaldehyde when spotted on to the plate, and fenchone gives an unreliable response to visualizing sprays (Betts, 1964). In addition, estragole has the same Rf value as anethole and is superimposed on it. Zacskó-Szász & Szász (1965) claim that estragole has a much lower Rf value than anethole, but their spray, phloroglucinol and hydrochloric acid, only detects an impurity. Gas chromatography was also required for quantitative work, as there is no very sensitive spectrophotometric assay for anethole. Peak height ratios were used, being more rapidly obtained, and giving the same result as peak areas: it was found that on any one occasion the peak width for a substance at half the peak height was constant.

Luyendijk (1957) and Betts (1965) observed that in caraway and dill fruits, carvone seems to reach a fairly constant level for each species some weeks before ripening. Although in bitter fennel there appears to be a "plateau" of development of both anethole and fenchone from about the fifth to seventh weeks, there is a subsequent increase in both constituents. Thus the results for both fennels do not suggest that the concept of a "specific level" is valid for the essential oil constituents of umbelliferous fruits in general, or even for the terpenoids. However, fennel fruits do ripen later, and take longer to do so, than those of caraway or dill, and this may account for the difference.

The anethole content of the two varieties of fennel is similar, although the fruits of the sweet variety are about twice the weight of those of the bitter variety, and so give a lower percentage oil yield. Anethole increases continually during fruit development, there being about 22 mg/100 fruits when they are ripe. By comparison with anethole peak areas, estragole also increases, but to less than 1 mg/100 fruits in both varieties. The fenchone content of the two varieties is different at all stages of fruit development, being much greater in the bitter variety than the sweet, and further increasing the percentage oil yield of the bitter variety. Fenchone forms about one-third of the combined anethole and fenchone content of the bitter variety, but only one-tenth or less of the combined constituents of sweet fennel, although it is definitely present at all stages of development (Table 1). Tóth (1967a) found that the oil of European bitter fennel fruit contained 12·3–22·2% fenchone, whilst that of European sweet fennel contained only 0·4–0·8%. There appears to be some disparity between Tóth's figures and the results obtained in the present work, where the percentage fenchone of both varieties was much higher, assuming that the essential oils were mainly anethole and fenchone. This may be due to partial decomposition of the fenchone during steam distillation, or to preferential loss of fenchone from the fruits before Tóth received

them* The latter is likely, as preferential loss of limonene relative to carvone occurs on storage of dill fruits (Kalitzki, 1954).

Both varieties of fennel, at all stages of their fruit development, contain some fenchone and therefore comply with what appears to be a B.P.C. requirement. However, the essential oil may contain over 20% fenchone or a negligible amount.

Acknowledgements are due to Mr. M. Humphrey of Bush, Boake, Allen Ltd. who kindly provided the packing for the gas chromatographic column and whose firm provided the reference anethole; to Dr. I. U. W. Osisiogu of Nigeria University, Nsukka for the reference sample of fenchone; and to Mr. L. Guglielmi who obtained the sweet fennel "seed" and raised the plants.

* Since completion of the present paper Tóth (1967b) has recorded a decrease in the fenchone content of fennel fruit stored for 10 months (along with an apparent (?) increase in anethole).

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On the specificity of the reversal of reserpine hypothermia for the evaluation of antidepressant effect

SIR,—The test to establish potential antidepressant activity based on the calorigenic effect in reserpinized animals has recently been considered unreliable (Whittle, 1967). Evidence was presented that compounds commonly considered not to have an antidepressant effect, such as chlorpromazine, aspirin and morphine were able to increase body temperature in mice previously made hypothermic by a large dose of reserpine.

Since experiments from this laboratory on interactions between tricyclic antidepressant agents and reserpine (Garattini & Jori, 1966; Bonaccorsi & Garrattini, 1966; Manara & Garattini, 1967) have always been made in rats we have repeated Whittle's experiments in this species.

Sprague-Dawley female rats weighing 150 ± 10 g were treated with reserpine (5 mg/kg i.v.) and 16 hr later were given drugs or solvent orally. Body temperature was measured before treatments and at regular intervals as much as 6 hr later by means of a thermistor. The experiment was at a room temperature of 20° with 56% relative humidity.

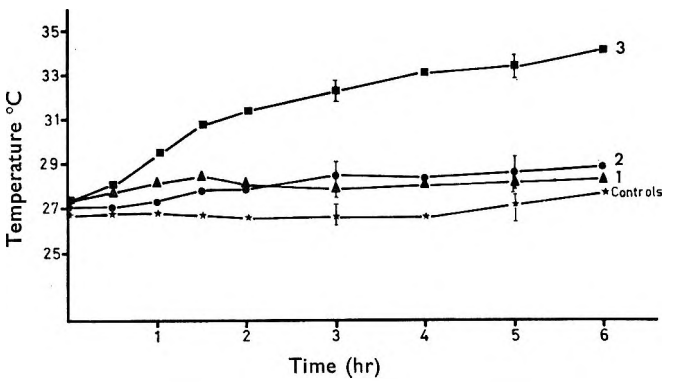

Fig. 1. Changes of body temperature induced by, (1) aspirin (300 mg/kg oral) (2) chlorpromazine (10 mg/kg oral) or (3) desipramine (3 mg/kg oral) in rats receiving reserpine (5 mg/kg i.v.) 16 hrs before the experiment. Vertical bars represent the standard error.

Fig. 1 shows that desipramine but not chlorpromazine or aspirin, induces a significant increase of body temperature in resperpinized rats. Our results with rats differ from those obtained by Whittle (1967) and by Morpurgo & Theobald (1965) (who tested chlorpromazine only) both using mice.

This discrepancy underlines the importance of species difference in comparing experiments and in extrapolating conclusions.

From the data available for rats it thus can be seen that the reversal of the reserpine-induced hypothermia allows a differentiation between tricyclic antidepressant agents and other classes of drugs to be made.

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N-(3-Benzylthio-2,6-dichlorophenyl)anthramyl acid (ASD 30): a non-competitive antagonist of bradykinin

SIR,—In 1966 methixene was reported to be a non-competitive antagonist of bradykinin (van Riezen, 1966). Recently Drs M. Taeschler and A. Fanchamps informed us that ASD 30 was a selective bradykinin antagonist. We have now examined the mechanism of action of this compound by the method used for methixene.

Guinea-pig ileum was bathed in a 10 ml bath with a Tyrode solution saturated with a mixture of oxygen 95% and carbon dioxide 5% at 37°. Two cumulative

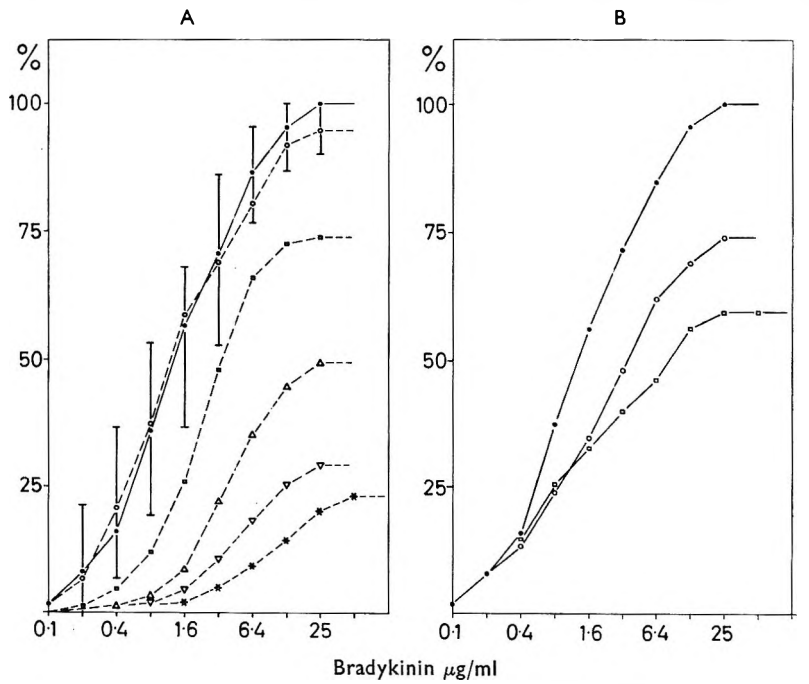

FIG. 1A. Cumulative dose response curves of guinea-pig ileum in Tyrode solution saturated with oxygen 95% and carbon dioxide 5% at 37° to bradykinin. With ASD 30 (mole/ml): ○- - - - -○ 1.10^{-6} (4); □- - - - -□ 3.10^{-6} (6); △- - - - -△ 1.10^{-5} (6); ▽- - - - -▽ 2.10^{-5} (2); *- - - - -* 5.10^{-5} (2). ●——● Control (20) 95% confidence limits. In parentheses: number of individual curves from which the curve is calculated.

B. In this experiment, the period of 30 min washing was followed by a second because the bradykinin curve was still below its control value. After this second wash the 1 hr curve ○——○ (2.10^{-5}mole/ml) was made. Then the preparation was again incubated with ASD 30 (2.10^{-5}mole/ml) for 20 min and the procedure repeated: curve □——□.

dose response curves with bradykinin were made and if these curves differed by less than 10% the experiment was begun. ASD 30 was dissolved by heating the required amount for one day's experimentation in 1–2 ml of N NaOH in a boiling water bath. This solution was then diluted with Tyrode to the required concentrations. The guinea-pig ileum was incubated with ASD 30 added to the medium to a final concentration of 1.10^{-6}, 3.10^{-6}, 1.10^{-5}, 2.10^{-5} or 5.10^{-5} mole/ml for 20 min, then cumulative dose response curves to bradykinin in the presence of the inhibitor were made. The guinea-pig ileum was then washed several times with fresh Tyrode solution during 30 min and a control cumulative dose response curve with bradykinin was made. The curves were readily reproducible. The same preparation was often used for more interaction curves whereas the same final inhibitor concentrations were tested in several preparations of guinea-pig ileum. A given dose was considered maximal if a two-fold higher dose did not induce an increased contraction of the preparation.

In Fig. 1A, cumulative dose-response curves to bradykinin (mean of 20 experiments) and of bradykinin in the presence of different concentrations of ASD 30 are shown. In the presence of 1.10^{-6}mole/ml ASD 30 no antagonism was seen. However, 3.10^{-6}mole/ml ASD 30 decreased the effect of bradykinin almost proportionately for each dose. This effect was more pronounced with the higher dose of inhibitor. The slope of the curves seemed to be dose-dependent, indicating a noncompetitive inhibitory mechanism. During the experiment with the doses of 2.10^{-5}mole/ml ASD 30 and higher, it became evident that the effect of ASD 30 was not completely reversible.

Fig. 1B shows the effect of 20 min incubation of guinea-pig ileum in Tyrode solution containing 2.10^{-6} mole/ml ASD 30. After a bradykinin response curve was made in the presence of the inhibitor, the preparation was washed several times during 1 hr with Tyrode solution without the inhibitor and a control bradykinin curve made. The sequence was then repeated a second time. It can be seen that even after 1 hr of washing, the bradykinin curve is only about 75% of the control. After another incubation period with the same concentration of ASD 30 the maximal response to bradykinin was 60%, after 1 hr of washing.

Thus ASD 30 seems to be a non-competitive bradykinin antagonist when tested on the guinea-pig ileum, the effect being only partially reversible in the higher dose ranges. The search for bradykinin antagonists has not yet uncovered a competitive bradykinin antagonist. Our method, being simple and rapid, might be useful in this search. The activity of bradykinin on the guinea-pig ileum does not seem to be related to the postulated role of the kinin as mediator of inflammatory and similar pathological conditions, and results from the ileum of the guinea-pig should be extrapolated with utmost reserve to other tissues.

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Acetylcholine release from the feline thalamus

SIR,—Thalamo-cortical relay neurons in the ventro-basal complex of the thalamus are excited by iontophoretically applied acetylcholine (Andersen & Curtis, 1964; McCance, Phillis & Westerman, 1966). Although the synaptic responses of these cells evoked by stimulation of limb nerves and the cerebral cortex are unaffected by acetylcholine antagonists, those initiated by stimulation of the mesencephalic reticular formation and brachium conjunctivum are reduced or abolished by atropine or dihydro-β-erythroidine (McCance, Phillis & Westerman, 1968). The suggestion that cholinergic pathways from the brain stem and cerebellum terminate on thalamic neurons (McCance & others, 1968) is supported by studies on the distribution of choline acetyltransferase and acetylcholinesterase in the stem and brachium conjunctivum (Feldberg & Vogt, 1948; Hebb & Silver, 1956; Shute & Lewis, 1963; Phillis, 1965).

To establish that a substance is a transmitter agent in a structure, it is desirable to demonstrate its presence in perfusates from the area during periods of physiological stimulation. For example, acetylcholine has been identified in perfusates from the cerebral cortex and caudate nucleus, and its rate of release correlated with variations in the amount of neuronal activation (Mitchell, 1963; Phillis & Chong, 1965; McLennan, 1964). The demonstration of a spontaneous release of acetylcholine from the thalamus, which can be augmented during periods of stimulation, would contribute to the confirmation of cholinergic transmission in this region of the central nervous system. A variety of modes of stimulation have been shown to cause an increase in the rate of release of acetycholine from the cerebral cortex, but as the increased release occurs in several cortical areas it is probably unrelated to the specific mode of stimulation employed. As stimulation of the reticular formation also causes an increase in cortical acetylcholine release (Kanai & Szerb, 1965; Phillis, 1968), it has been suggested that projections of the reticular arousal system are cholinergic and that the increased release observed during stimulation of peripheral structures is probably a result of activation of this system (Phillis, 1968). In the present investigation, we have shown that stimulation of various forms of input causes similar increases in release of acetylcholine from the thalamus.

Nine adult cats were used; two of these were anaesthetized with pentobarbitone sodium and in the remaining seven, anaesthesia was induced with thiopentone sodium and maintained by a gas mixture of nitrous oxide, oxygen and methoxyflurane (Penthrane, Abbott). The animals were mounted in a stereotaxic frame and after removal of the cranial vault, cortical and subcortical tissue of the left cerebral hemisphere overlying the thalamus was excised by suction to expose the hippocampal fornix and fimbria and the floor of the fourth ventricle between stereotaxic co-ordinates A7–A12 and midline to L9. A push-pull cannula (Gaddum, 1961) was inserted into the thalamus at co-ordinates A9, L5. The amounts of spontaneous release were ascertained at two depths (3 and 8 mm) below the surface of the fornix. Stimulation was employed only when the cannula tip was in the ventro-basal complex. The position of the cannula tip was verified histologically at the termination of each experiment.

Each push-pull cannula was fabricated from 27 and 18 SWG hypodermic syringe needles and mammalian physiological saline (for composition see Phillis, 1968) and was circulated through both needles by a Braun Unita II, two channel push-pull infusion pump, fitted with matching syringes. Dead space in the collecting tube was 0·1 ml, representing 10% of the standard sample volume (10 min collection period and a flow rate of 0·1 ml per min). Before sample collection, the area was perfused with a 2×10^{-5} g/ml neostigmine solution for 30

min and a similar concentration of neostigmine was present in the solutions for assay. The samples were assayed on the heart of *Tapes watlingi* (Chong & Phillis, 1965), each sample being assayed on two hearts in small (0·3 ml) Perspex baths. The inhibitory activity in the samples was abolished by the acetylcholine antagonist, benzoquinonium, or by boiling the samples briefly in an alkaline solution. The hearts were exposed to the 5-hydroxytryptamine antagonist, methysergide (UML 491) before and during assays.

Stainless steel pins were inserted into the contra- and ipsi-lateral forepaws for limb nerve stimulation, a bipolar coaxial stimulating electrode at stereotaxic co-ordinates A3, L3, D-1 was used to stimulate the reticular formation and a Grass PS 2 photostimulator provided light flashes for visual stimulation.

A spontaneous release of acetylcholine was observed in all animals at both dorsal and ventral positions in the thalamus. The rates of release were substantially reduced (50–200 pg/min) in the two animals anaesthetized with pentobarbitone sodium in comparison with the gas anaesthetized animals (100–600 pg/min). There was a marked tendency for the initial one or two samples to contain higher levels of acetycholine than those immediately following them, which suggests that implantation of a push-pull cannula may be attended by damage to nerve cells and endings with a resultant leakage of acetycholine from the fragmented tissues. After periods of stimulation, the rates of spontaneous release usually continued at an elevated level. The rates of release from both superficial and deep thalamic areas appeared to be comparable.

The three forms of stimulation all caused an increase in the rates of release of acetylcholine. In Fig. 1 are histograms illustrating the effects of visual (light flashes, A), limb, (B) and mesencephalic reticular formation (C) stimulation on the rate of release of acetylcholine from the ventro-basal complex of the

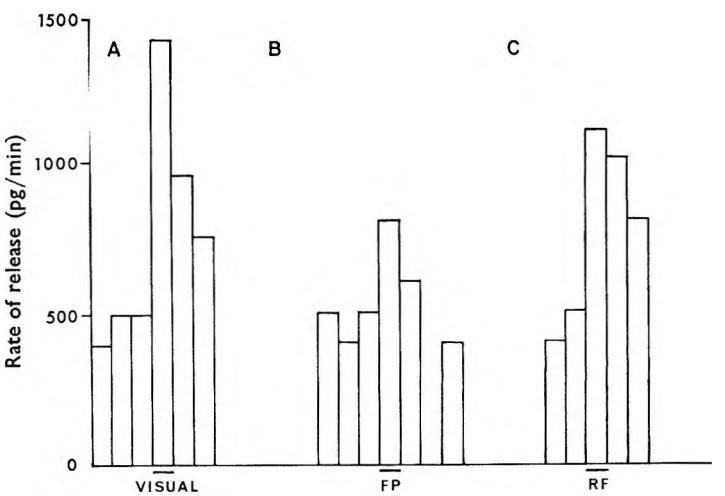

FIG. 1. A, B and C are histograms showing rate of acetycholine release (pg/min) from the ventro-basal thalamic complex of three preparations, before, during and after stimulation. Each division of abscissa represents one 10 min collection period. A: effects of visual stimulation (1/sec). B: effects of contralateral forepaw (FP) stimulation (1/sec). There was a 10 min gap between collection of the last two samples. C: effects of stimulating the mesencephalic reticular formation (RF) (2/sec). Position of the stimulating electrode was verified histologically.

thalamus. In A, the resting release of acetylcholine was 400–500 pg/min. Visual stimulation (1/sec) caused an increase to 1400 pg/min during the period of stimulation and this rate declined slowly during the subsequent collection periods. Fig. 1B shows that stimulation of the contralateral forepaw (1/sec) elevated the rate of release from 400–500 pg/min to 800 pg/min. Similarly, stimulation of the reticular formation doubled the rate of release of acetylcholine (Fig. 1C).

The effects of stimulation were frequently less marked than those demonstrated in Fig. 1 and if the initial period of stimulation resulted in a marked elevation of the rate of spontaneous release, subsequent periods of stimulation were invariably less effective. A similar observation has been made in experiments on the cerebral cortex (Phillis, 1968).

The results described support the hypothesis that acetylcholine is a synaptic transmitter in the feline thalamus (McCance & others, 1968). As the medial lemniscal pathway conveying afferent volleys from limb nerves is unlikely to be cholinergic (Andersen & Curtis, 1964; McCance & others, 1968), increased thalamic release of acetylcholine following limb nerve stimulation may be a result of activation of the reticular arousal system. A similar conclusion can be drawn from the increased acetylcholine release evoked by visual stimulation. Such conclusions are strengthened by the finding that reticular formation stimulation itself causes an increase in the rate of release and that synaptic activation of thalamic neurons by brain stem stimulation is abolished by antagonists of acetylcholine (McCance & others, 1968). The finding that acetylcholine release from the dorsal thalamus was comparable to that from the ventro-basal complex is more difficult to reconcile with studies on the distribution of acetylcholine-excited neurons in the thalamus, as they are predominantly located in the ventro-basal complex. However, acetylcholine inhibition of neurons in the dorsal thalamus has also been described (McCance & others, 1968) and the release from this area may be related to inhibitory cholinergic pathways.

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Inhibition of red cell agglutination in the ABO system by promethazine

SIR,—We observed that the addition of promethazine hydrochloride to normal human serum delayed the rate of agglutination of red cells containing the corresponding antigens, particularly in the ABO blood group system (Barrie & Tait, 1967). Since promethazine crosses the placental barrier, it is possible that if administered during pregnancy it might prevent cell destruction in the foetus in the case of blood group incompatibility between mother and infant. Biermé & Biermé (1967), in fact, have reported that promethazine had been used with apparent success in conjunction with intraperitoneal transfusion in the treatment of hydrops foetalis due to Rh incompatibility. I have therefore examined further the effect of promethazine on red cell agglutination.

Because high concentrations of promethazine cause haemolysis of erythrocytes (Seeman & Weinstein, 1966), preliminary tests were made to determine the concentration producing haemolysis. A sample of 0·2 ml of a suspension of Group A red cells in 0·9% saline was added to 3·8 ml of distilled water and the haemolysed mixture was adjusted to give a colorimeter (EEL) reading of 3·5, corresponding to a haemoglobin value of 1·02 g/100 ml suspension. Equal volumes (0·75 ml) of drug solution (10^{-1} to 10^{-4} M in 0·9% saline) and of Group A cell suspensions were mixed in haemagglutination tubes (9 cm × 0·7 cm) and allowed to stand at 20° for 30 min. After centrifugation, the haemoglobin content of 1 ml of supernatant was measured spectrophotometrically and units of haemoglobin read from a standard curve obtained from cells of known haemoglobin content (Red Cross Blood Transfusion Service, Sydney). The minimum final concentration of promethazine producing haemolysis was between 0·125 and 0·25 × 10^{-2} M. Using serum in place of saline gave a slightly higher end point (0·25 — 0·375 × 10^{-2} M) possibly due to a lower fragility of red cells in serum. The haemolytic effect of promethazine was independent of concentration of red cells in suspensions ranging from 2 to 10%.

The effects of promethazine on agglutination were investigated in a system consisting of Group A or Group B red cell suspensions standardized to a haemoglobin value of 1·02 g/100 ml and mixed Group O serum standardized to contain approximately constant anti-A and anti-B titres. Solutions of promethazine in 0·9% saline containing 0·75 × 10^{-2}, 3 × 10^{-3}, 3 × 10^{-4}, 3 × 10^{-5} and 3 × 10^{-6} M of drug were prepared. Drug solution (0·25 ml) was added to 0·50 ml of serum and mixed thoroughly with 0·75 ml of cell suspension in a haemagglutination tube. Control tests were made with undiluted serum and with 0·9% saline. The tubes were stood for 30 min at 15–20° during which time the agglutinated cells settled. The proportion of agglutinated cells was determined by measuring the haemoglobin content of the supernatant which contained the unagglutinated cells. An aliquot of 1 ml of the upper portion of supernatant was removed (without centrifugation) and was added to 4 ml of distilled water to haemolyse the red cells present and the haemoglobin value was estimated spectrophotometrically. The addition of promethazine to serum invariably produced a fine white precipitate. Samples were therefore filtered immediately before taking spectrophotometric readings.

The percentage of agglutinated cells are plotted against promethazine concentration in Fig. 1. The highest concentration used was 1·25 × 10^{-3} M, which was approximately the concentration causing the first appearance of haemolysis. This suggests that without the interference of haemolysis even greater inhibition of agglutination may have occurred with higher concentrations. The threshold concentration of promethazine for inhibition of agglutination was 5 × 10^{-5} M. Similar results were obtained when solutions of promethazine

were prepared from ampoules (25 mg/ml). Since saline solutions of promethazine hydrochloride (pure substance) rapidly deteriorated on standing in the light, it was more convenient to use the ampouled drug.

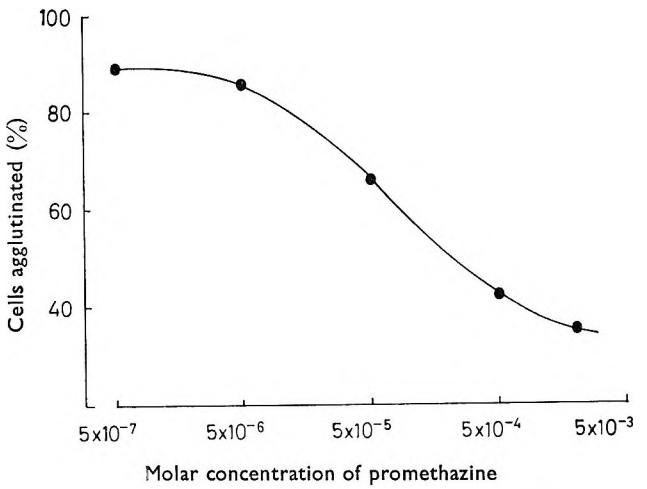

FIG. 1. The range of concentration of promethazine producing inhibition of agglutination of a Group A cell suspension by Group O serum. Molar concentration of promethazine is shown on the horizontal axis. The threshold for haemolysis of cells by promethazine (approximately $1{\cdot}25 \times 10^{-3}$ M) set an upper limit on the concentration used. The percentage of cells agglutinated is plotted on the vertical axis.

The inhibition of agglutination produced by promethazine ($1{\cdot}25 \times 10^{-3}$ M) was not influenced by the group of the red cells used. Thus in 6 samples with Group A cells the mean haemoglobin content of the supernatant was 0·196 g/100 ml, whereas with 6 samples of Group B cells it was 0·184 g/100 ml. The difference was not significant (*t*-test: $t = 0{\cdot}16$, $P = 0{\cdot}8$).

Since the estimation of agglutination depended on the measurement of the haemoglobin value of cells remaining in suspension, it was important to ascertain whether promethazine affected the sedimentation rate of fresh cells. Accordingly, observations were made on suspensions of Group O cells with Group O serum, together with saline or promethazine solutions and these mixtures were allowed to stand for 30 to 75 min. No significant difference was detected between the sedimentation rates of cells in the various samples. This result agrees with that reported by Shohl & Schmidt (1959). Steinbuch (1953) found that promethazine decreased the sedimentation rate in fresh blood but accelerated it in blood stored for more than four days. The cells used in these experiments were from freshly drawn blood (less than 24 hr old).

The inhibitory effect of promethazine on agglutination appears to be in the nature of a delaying factor and not a complete inhibition, since in qualitative tests performed on a slide rather than in a tube the cells appeared to agglutinate normally after a prolonged period of incubation at room temperature (15–20°). It was noted that when the control samples exhibited a high degree of agglutination, the inhibitory effect of promethazine was more marked.

Acknowledgement. The author is greatly indebted to May and Baker Ltd. who generously supplied promethazine hydrochloride in prepared solution and purified powder form.

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Gas-liquid chromatography of imidazoline salts

SIR,—A gas-liquid chromatographic method for analysis of imidazolines in pharmaceutical preparations has recently been published by Boon & Sudds (1967). Their treatment included both antazoline and naphazoline in the group of five heterocyclics investigated. These two compounds may be conveniently analyzed by a procedure which offers the advantages of reduced tailing and inclusion of an internal standard.

A dual column flame ionization chromatograph was operated under the following conditions: flash heater at 250°, column temperature at 239°, isothermal; detector temperature at 265°; helium carrier gas at 40 p.s.i.g. and 70 cc/min; hydrogen at 60 cc/min; air at 300 cc/min; and a recorder range of 10 with attenuation of 32 for carbazole and naphazoline and 64 for antazoline. These recorder adjustments are stated for use of manual quantization. Results were obtained using an integrator. The columns consist of 4 ft of 6 mm O.D. U-shaped glass tubing containing Chrom-Q, 100–120 mesh, treated with 1·0% KOH in methanol and coated with 5·0% of Apiezon-L using methylene chloride as the solvent.

Procedure. Add an aliquot of sample containing approximately 4–40 mg each of naphazoline salt and antazoline salt to a separatory funnel. Make the solution basic with sodium hydroxide and extract immediately with chloroform. Combine the chloroform extracts and add a portion of the internal standard solution containing approximately 4 mg of carbazole in chloroform. Evaporate to approximately 2 ml and inject 0·5 to 1·2 μl of the solution. Calibrate the sample by comparing the ratio of the peak areas of each imidazoline to the peak area of the internal standard.

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Potentiation of methamphetamine aggregate toxicity in mice by diethyldithiocarbamate

SIR,—Diethyldithiocarbamate (DDTC), a metabolite of disulfiram, inhibits the enzyme dopamine-β-hydroxylase, causing an increase in dopamine and a decrease of noradrenaline in mouse brain (Hashimoto, Ohi & Imaizumi, 1965). It has been postulated that dopamine release is a mechanism for amphetamine-induced locomotor stimulation and stereotyped behaviour (Rossum & Hurkmans, 1964; Randrup & Jonas, 1967). If dopamine release also is involved in methamphetamine-induced aggregate toxicity, DDTC should potentiate the effects of submaximal doses of methamphetamine.

Groups of 10 male CF # 1 mice, 18–22 g, were housed in covered plastic cages (30 cm × 15 cm × 13 cm). The LD50 for methamphetamine aggregate lethality was 6 mg/kg, intrapenitoneally, and this dose was used in all experiments. DDTC was administered intraperitoneally 30 min before methamphetamine and deaths were counted 24 hr later.

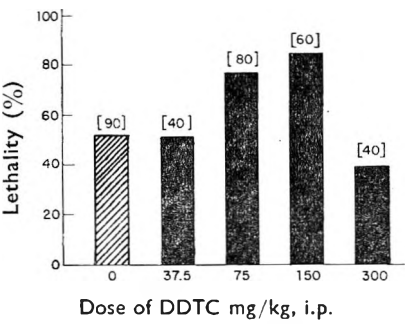

FIG. 1. The influence of DDTC on the aggregate lethality produced by methamphetamine, 6 mg/kg, i.p. Total number of mice used for each dose of DDTC is in brackets.

As shown in Fig. 1, DDTC, 75 and 150 mg/kg, significantly *increased* the aggregate lethality of methamphetamine. The levels of significance as determined by the Fisher Exact Probability test were $P < 0.005$ and $P < 0.001$, respectively (Siegel, 1956). These doses of DDTC (75 and 150 mg/kg) do not affect overt behaviour in mice and are well below its LD50 (1500 mg/kg) (Hald, Jacobsen & Larsen, 1952). DDTC 300 mg/kg markedly depressed spontaneous motor activity in normal mice, which may explain why further potentiation of methamphetamine was not seen at this dose.

DDTC (75 and 150 mg/kg), which significantly increased methamphetamine lethality, also reverses reserpine-induced hypothermia (Barnett & Taber, 1968); whereas, DDTC, 37·5 mg/kg, was ineffective in both procedures. These two effects of DDTC can be explained by its ability to increase brain dopamine content.

It has been postulated that the cause of amphetamine aggregate lethality is increased motor activity (Greenblatt & Osterberg, 1961) or hyperthermia (Greenblatt & Osterberg, 1961; Menon & Dandiya, 1967). Since DDTC (75–300 mg/kg) produces hypothermia in normal mice (Barnett & Taber, 1968), it seems likely that it potentiates methamphetamine aggregate lethality by increasing the locomotor stimulant rather than the hyperthermic effects of methamphetamine.

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Crystallization in polyhedral emulsion particles

SIR,—During an investigation of the properties of emulsions, preparations were made by adding a mixture of cetyl alcohol in liquid paraffin at 65° to an aqueous solution of sodium dodecyl sulphate at the same temperature and stirring with a Silverson mixer until cold. The systems used were liquid paraffin 100 g, water 300 g and:

	A	B	C	D	E	F	G	H	I
Sodium dodecyl sulphate ..	0·8	1·2	1·6	2·0	2·4	2·8	3·2	3·6	4·0 g
Cetyl alcohol ..	7·2	10·8	14·4	18·0	21·6	25·2	28·8	32·4	36·0 g

Non-spherical particles were observed microscopically in each cooled emulsion, even though the concentration of alcohol in liquid paraffin was sufficiently low to ensure that the globules were essentially liquid. These deformed globules varied in shape from flattened spheres to polyhedra, and contained anisotropic crystals which lay along the straight sides of the globules. Crystals were formed even though the alcohol to liquid paraffin ratio was much less than 8:5, which has been quoted as the minimum ratio for crystallization in similar systems by Groves & Scarlett (1965). The crystals I observed were usually acicular but occasionally flat and hexagonal. They were not easily visible in ordinary light but showed up clearly when a specimen was mounted between crossed polars. The system of lowest emulsifier concentration (A) contained some large globules

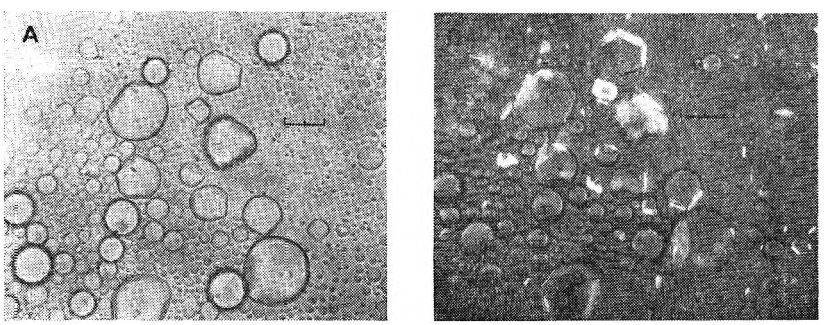

FIG. 1. Photomicrographs of System A mounted in 50% v/v glycerol in water. (a) Ordinary light (b) crossed polars. One division = 10 μ.

which showed this phenomenon well (Fig. 1). Thus it would appear that in these systems the formation of polyhedral particles is a result of crystallization.

Recently, there have been reports of irregular polyhedral particles in semi-solid emulsions consisting of equal parts by weight of cetostearyl alcohol and liquid paraffin dispersed in 0·5% w/w aqueous cetrimide solution and prepared in a similar manner to the above (Groves & Scarlett, 1965, Groves & Freshwater, 1967). It was concluded that during the emulsification process localized close-packing existed, droplets were distorted and polyhedral particles were formed. As these were solid at room temperature, their shapes were maintained on cooling and on dilution for microscopic examination. In other cases, it was suggested that liquid particles may retain their shapes because of the rigidity of the interfacial film. In both papers it was concluded that the polyhedral particles were unlikely to be due to crystallization of the alcohol. However, cetrimide emulsions prepared to the above formula have been examined in this laboratory and crystals have been detected in deformed globules. Thus, crystallization as a cause of polyhedral emulsion particles in these systems cannot be discounted.

Preparation for microscopy may also affect the behaviour. The examination of emulsions for polyhedral particles usually requires a dilution which in the first instance should be made with a surfactant solution of the same strength as that used in the preparation of the emulsion. This avoids a change in the nature of the continuous phase which may increase crystallization. Thus, with systems A to I and the cetrimide emulsions, dilution with 50% v/v glycerol in water caused a small increase in the fraction of globules containing crystals; when mounted in undiluted glycerol, crystals were precipitated in most of the larger globules. It may be convenient to mount specimens in aqueous glycerol solutions for photomicroscopy as in Fig. 1, so as to reduce Brownian movement, but if the fraction of globules which are polyhedral or contain crystals is to be estimated, it is necessary to confirm that the mounting technique does not increase the proportion.

With time, systems A to I changed in appearance as they became more mobile and formed silvery crystalline deposits in the external phase. The lower the concentrations of the mixed emulsifiers the more readily deposits were formed. These anisotropic, hexagonal, flat crystals melted *in situ* at temperatures varying between 28° and 39° (determined using a Kofler Micro Hot Stage). Similar crystals have been reported for the sodium dodecyl sulphate—cetyl alcohol—water system (Barry & Shotton, 1967). They should not be confused with polyhedral emulsion droplets.

Acknowledgement. The author wishes to thank Mr. J. Mauger for taking the photographs.

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Programmed kinetic studies

SIR,—Recently Cole & Leadbeater (1966) described a detailed evaluation of the "non-isothermal kinetics" test proposed by Rogers (1963) and Eriksen & Stelmach (1965). This test allows calculation of the complete rate equation (i.e., $\log k = f(1/T)$, k being the rate constant of a homogeneous degradation, T being the absolute temperature) in a single experiment. The single-step equation for first order cases, using the nomenclature of Cole and Leadbeater is

$$\log\left(\ln\frac{c_0}{c_t}\right) = \log k_0 - \log\left(1 + \frac{EB}{R}\right) + \left(1 + \frac{EB}{R}\right)\log(1 + t) + \log\left(1 - \left[\frac{k_0}{k_t}\right]^{1+\frac{R}{EB}}\right) \quad .. \quad .. \quad 1$$

and is derived from the relation

$$\frac{d(\log k)}{d(1/T)} = -\frac{E}{2{\cdot}3\,R} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad 2$$

and the programming function

$$\frac{1}{T_0} - \frac{1}{T_t} = B \ln(1 + t) \quad .. \quad .. \quad .. \quad .. \quad .. \quad 3$$

The authors point out precautions to be taken, suggest preliminary experiments where needed (order of reaction) and call attention to the assumptions made by the originators of the test.

A few further comments may be added for the benefit of those who plan on using "non-isothermal kinetics" as tool for stability predictions.

The case sometimes occurs where distinct, non-zero equilibria eventually are reached, and in these situations, the theory underlying the linear programming leads to equations which cannot be made linear. The simplest example (Carstensen, 1968) of equilibrium which may be visualized is $A \rightleftharpoons B$, where A is the parent compound and B the degradation product. If A_0 is the initial concentration, x the fraction decomposed, and k_+ and k_- the forward and reverse rate constants, then the rate equation

$$\frac{dx}{dt} = k_+(1 - x) - k_-x \quad .. \quad .. \quad .. \quad .. \quad .. \quad 4$$

after rearrangement and invokement of initial conditions integrates to

$$x = x_e(1 - e^{-(k_+ + k_-)t}) \quad .. \quad .. \quad .. \quad .. \quad .. \quad 5$$

where the equilibrium fraction is $x_e = \frac{k_+}{k_+ + k_-}$; although this may be made linear in isothermal experiments, e.g. by following $\log \frac{A_0(1 - x)}{A_0(1 - x_e)}$ (i.e. the logarithm of the amount of drug remaining, divided by the amount at "infinite time") as a function of time, the equilibrium concentration $A_0(1 - x_e)$ changes with temperature by a van't Hoff relationship. Where equation 5 applies, equation 2 (at constant pressure) would take the forms:

$$\frac{d\,(\log k_+)}{d\,(1/T)} = -\frac{E_a}{2{\cdot}3\,R} \qquad 6$$

and

$$\frac{d\,(\log k_-)}{d\,(1/T)} = -\frac{E_a + H}{2{\cdot}3\,R} \qquad 7$$

where H is the heat of reaction. The only measurable parameter would be $(k_+ + k_-)$, and there is no way of expressing $d(\log(k_+ + k_-))/d(1/T)$ in manageable analytical form. The actual effect is that a_∞ in the original paper of Rogers (1963), apart from not representing zero concentration, is not time-independent (temperature-independent). Similar arguments hold for the slightly different programming $\left(\frac{1}{T_o} - \frac{1}{T_t} = a\,t\right)$ employed by Eriksen & Stelmach (1965).

Apart from this limitation, it should be borne in mind that whenever a kinetic salt effect exists (see e.g. Garrett, 1958), linearity might be lost. If the experiment is conducted at a particular ionic strength (by using buffers) which is usually the case, then the kinetic salt effect will change with temperature, since the factor to $2\,z_+\,z_-\sqrt{\mu}$ is

$$1.825{\cdot}10^6\left[\frac{\rho}{\epsilon^3 T^3}\right]^{\frac{1}{2}}$$

where ρ denotes density, ϵ dielectric constant, T absolute temperature, z charge, and μ ionic strength. For example, the value of this coefficient for water is 0·509 at 25° and 0·468 at 60° (Harned & Owen, 1954). It should be noted that the factor does not vary proportionally to T^{-1} but to $T^{-3/2}$. The temperature dependency of the dielectric constant further complicates the behaviour as the temperature is increased. Since the factor difference at two temperatures equals the logarithm of the ratio of rate constants (over and above the inverse temperature effect) the contribution can be sizable. It is even more pronounced in systems which are not completely aqueous (e.g. 50 : 50 acetone water), and have smaller and more temperature sensitive dielectric constants. Therefore, it would be advisable always to check for kinetic salt effect before conducting single-step stability studies.

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Effects of reserpine on the plasma half-time of [^{131}I]thyroxine

SIR,—Canary, Schaaf & others (1957), using reserpine, relieved the signs and symptoms of patients with thyrotoxicosis without influencing thyroidal [^{131}I]-uptake or the serum protein-bound iodine level. Although conflicting studies have been reported, reserpine probably has no direct effect upon thyroid-stimulating hormone (TSH) release, or the thyroidal metabolism of iodine (Watts, 1967). These reports led to the consideration that reserpine might have a direct peripheral anti-thyroxine effect.

Male Sprague-Dawley rats weighing 165–175 g were placed on a low iodine diet and divided into three groups. The animals in group A received 100 μg/kg of reserpine intraperitoneally daily for three days. They were then given 0·1 μg [^{131}I]thyroxine, initially containing 20·7 μc/μg, intraperitoneally. The same dose of resperpine was continued until the animals were killed. Group B received a single injection of 2 mg reserpine intraperitoneally 2 hr before receiving the [^{131}I]thyroxine. These doses of reserpine did not influence food intake. Group C received only the [^{131}I]thyroxine. Heparinized blood samples were taken from the tail at 6, 24, 48 and 72 hr after [^{131}I]thyroxine injection, and at 96 hr by direct cardiac puncture.

The plasma was subjected to paper chromatography (Taurog, Tong & Chaikoff, 1950), and the radioactivity of the thyroxine band was determined in a scintillation well-counter. By plotting these values (in counts/ml plasma) on semi-logarithmic paper and extrapolating back to zero time, the half-time of the plasma [^{131}I] thyroxine was obtained. At the time of death, the brain was removed and its 5-hydroxytryptamine (5-HT) content determined (Mead & Finger, 1961).

The control animals (Group C) yielded a plasma thyroxine half-time of 23 $\pm$ 0·3 hr, whereas the half-times in the acute (Group B) and the chronic (Group A) reserpine experiments were 23 $\pm$ 0·7 and 23 $\pm$ 1·0 hr respectively. The average brain 5-HT content from Group A was 0·33 $\pm$ 0·02 μg/g brain. One animal taken at random from Group C yielded a brain 5-HT content of 0·45 μg/g whereas one taken at random from Group B had a brain 5-HT content of 0·26 μg/g.

Reserpine did not seem to alter the plasma half-time of [^{131}I]thyroxine in doses which significantly lowered brain 5-HT content ($P < 0·01$). Preliminary studies had demonstrated that the technique used for measuring thyroxine half-time was sufficiently sensitive enough to reveal a shortening of the half-time in unshorn rats at 4° by 25% and in rats given TSH by almost 50%.

Brodie, Davies & others (1966) reported that thyroid hormone increased the amounts of adenyl cyclase in adipose tissue. Catecholamines, by activating adenyl cyclase, increase the steady-state level of adenosine-3′,5′-phosphate which may be the chemical trigger mediating catecholamine action in sympathetic target organs (Sutherland & Rall, 1960). Therefore, since reserpine depletes catecholamines peripherally (Brodie, Olin & others, 1957), it may indirectly inhibit thyroid hormone function peripherally, without affecting its degradation, by reducing the availability of catecholamines, and thus limiting the activation of adenyl cyclase. This may explain why Canary & others (1957) noted relief of the signs and symptoms of patients with thyrotoxicosis who were treated with reserpine without noting any change in thyroidal [^{131}I]-uptake or in the serum protein-bound iodine level. Reserpine might be a useful agent in further studies of possible functional relations between peripheral thyroid hormone and catecholamines.

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Effect of HC-3 on choline uptake by the isolated diaphragm

SIR,—It is generally accepted that the action of HC-3 reaches a maximum only at stimulation rates of 1/sec or more (Wilson & Long, 1959). This is believed to result from impairment of acetylcholine synthesis which becomes more important at higher frequencies of stimulation leading to depletion of transmitter stores. However, choline does not antagonize HC-3 blockade in the rat phrenic nerve-diaphragm preparation (Thies & Brooks, 1961), and the addition of choline to fluid bathing the diaphragm fails to increase acetylcholine output (Straughan, 1960). Thus, the supply of choline may not be a limiting factor in acetylcholine synthesis in the diaphragm as in the superior sympathetic ganglion (MacIntosh, 1963). While investigating [^{14}C]choline uptake in rabbit isolated, perfused hearts, it was of interest to examine [^{14}C]choline uptake by the rabbit diaphragm and the influence of HC-3 thereon.

The preparation consisted of the rabbit isolated, perfused phrenic nerve-diaphragm as first described by Burgen, Dickens & Zatwas (1949) and later modified for continuous perfusion by Dr. L. P. McCarty (personal communication). The diaphragms were perfused with a re-cycling system via the vena cava with oxygenated, eserinized Locke-Ringer solution, 37°, 5 ml/min, while suspended in a Locke-Ringer bath. Both phrenic nerves were placed over platinum electrodes and monophasic pulses of 0·5 msec duration and 5 V were delivered from a Grass 54B stimulator. The frequencies of stimulation ranged from 6/min to 10/sec and were administered for 5 min followed by 5 min rest. This intermittent stimulation was continued for 1 hr. The concentration of [^{14}C]choline in the perfusion fluid was 0·012 μg/ml. Extraction of [^{14}C]-labelled compounds and their subsequent paper chromatography are as previously described (Buterbaugh & Spratt, 1968).

The results obtained are shown in Fig. 1. The response to nerve stimulation was maintained throughout the 1 hr perfusion period at frequencies of 6/min and 1/sec. At 10/sec, the contraction response was vigorous at the start of each 5 min period and diminished to about 25% of the initial response by the end of each period. Higher stimulation frequencies resulted in tetany and were not used. It is evident that the highest uptake of [^{14}C]choline occurred at the lowest stimulation frequency, 6/min, and decreased to the lowest value at 10/sec.

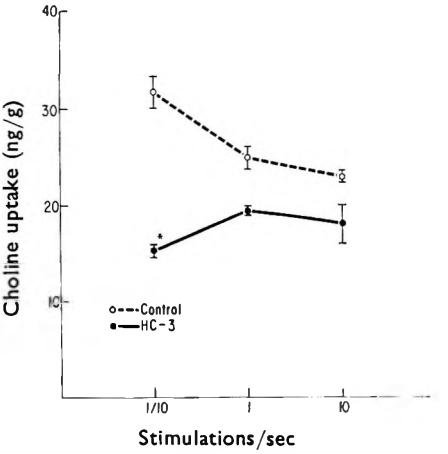

FIG. 1. [^{14}C]Choline uptake by isolated, perfused phrenic nerve-diaphragm as a function of stimulation frequency. Each point represents the mean ± s.e. for four hearts. Asterisk indicates value significantly different at $P < 0.05$ (Student's *t*, unpaired, two-tailed).

The significance of this observation is open to speculation. Perhaps it is evidence of a re-uptake mechanism for choline or acetylcholine at the presynaptic membrane which becomes of importance only at higher stimulation frequencies. [^{14}C]Acetylcholine could be extracted from the tissue in amounts of not more than 5% of the [^{14}C]choline uptake. However, it cannot be concluded that this [^{14}C]acetylcholine is synthesized or stored within the nerve endings since MacIntosh has concluded that over one half of the acetylcholine in leg muscle is located in non neural tissue (MacIntosh, 1963).

HC-3 added to the perfusion fluid in concentrations of 50 μg/ml had little effect on the contraction response at stimulation frequencies of 6/min and 1/sec. At 10/sec, the response was diminished to less than 25% of the initial response after the first 5 min of stimulation and did not recover. A significantly greater effect of HC-3 on [^{14}C]choline uptake was observed at the lowest stimulation frequency, 6/min, and no significant effect on choline uptake was seen at the higher stimulation frequencies of 1/sec and 10/sec. HC-3 had no effect on the level of [^{14}C]acetylcholine extracted from the tissue.

These results raise further questions directed toward cholinergic mechanisms. The action of HC-3 to produce a greater blockade of choline uptake at lower stimulation frequencies is contrary to commonly accepted facts concerning the action of HC-3 and emphasizes the uniqueness of this compound and the need for continued research on its mechanism of action.

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Modified method for the estimation of metaraminol and α-methyl-*m*-tyramine

SIR,—In recent years the catecholamine-depleting action of DL-α-methyl-*m*-tyrosine has attracted attention. Much evidence exists to suggest that the depleting agent is metaraminol, which is formed *in vivo* from methyltyrosine* via the intermediate α-methyl-*m*-tyramine (Carlsson & Lindquist, 1962). It has been postulated that metaraminol, which is taken up and stored within sympathetic nerves, may replace the noradrenaline and serve as a false transmitter (Crout, Alpers & others, 1964; Andén, 1964).

The *o*-phthalaldehyde method for the determination of metaraminol (Shore & Alpers, 1964) is specific for primary *m*-hydroxyphenylethyl amines, but will also produce fluorescent reactions with α-methyl-*m*-tyrosine and α-methyl-*m*-tyramine. It is therefore necessary to separate the three substances before condensation with *o*-phthalaldehyde. This is possible by passing the compounds through a Dowex-50 resin which allows the methyltyrosine to run through while retaining both the methyltyramine and metaraminol. Subsequent differential elution with N and 2N HCl allows a separation of the latter two amines.

We wish to report a method based on the above principles, sufficiently sensitive to allow for the analysis of single rat hearts. Columns of Dowex 50W, X-4, 450 mm by 60 mm, are prepared with sodium hydroxide, hydrochloric acid and 0·1M phosphate buffer, pH 6·5, as described by Carlsson & Lindquist (1962).

Single hearts, removed from rats treated with the methyltyrosine, are homogenized at high speed (VirTis "23" Homogenizer) in 25 ml of 0·4N perchloric acid. The supernatant is neutralized with cold 5N potassium carbonate and the ensuing precipitate removed by centrifugation (8000 rev/min for 10 min). The total amount of the supernatant remaining (approx. 25 ml) is forced through the Dowex column at a rate of 17 drops/min. The column is then washed with 10 ml of double distilled water and eluted first with 25 ml of 1N HCl and then with 25 ml of 2N HCl. The eluate is collected in 2·5 ml fractions which are then condensed with *o*-phthalaldehyde according to Shore & Alpers (1964).

The above method makes it possible to separately elute metaraminol and the methyltyramine (Fig. 1). The first curve, seen after the injection of the methyltyrosine or metaraminol, is metaraminol, and the second curve, observed only after methyltyrosine administration, is methyltyramine. Either curve can be selectively produced by the addition respectively of metaraminol or methyltyramine to extracts of hearts removed from untreated rats.

Quantitative estimation of the levels of metaraminol and methyltyramine present in the hearts is routinely obtained by summing the concentrations measured in eluates 3 to 8 inclusive, for metaraminol, and 12 to 19 inclusive for methyltyramine. We found the percentage recovery using this method to be 60·6 ± 5·3 for metaraminol and 62·8 ± 4·6 for methyltyramine.

* In this text methyltyrosine is used as an abbreviation for DL-α-methyl-*m*-tyrosine and methyltyramine for α-methyl-*m*-tyramine.

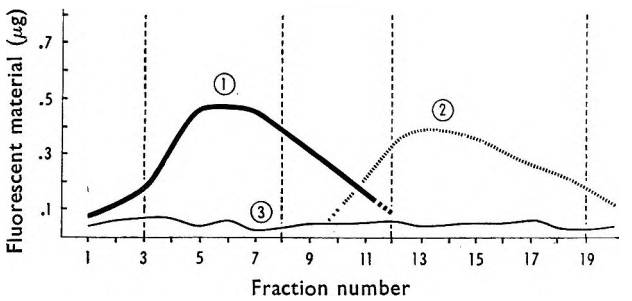

FIG. 1. Elution curves showing the separation of metaraminol (curve 1) and α-methyl-*m*-tyramine (curve 2) after the addition of 3·3 μg of each to extracts of hearts from untreated rats. Curve 3 was obtained from control hearts. Curves represent means of 6 analyses.

The procedure reported here involves principles previously employed (Andén, 1964). However, it differs from the previous method by describing the patterns of elution for metaraminol and methyltyramine and thereby demonstrating the feasibility of separating the two amines by differential elution. In addition, the sensitivity of the method reported here allows for the analysis of single hearts instead of pooled hearts. Results compare closely with earlier published values from pooled organs. For example, 4 hr after injection 400 mg/kg of DL-α-methyl-*m*-tyrosine we measured 2·5 nmole of metaraminol and 2·2 nmole of methyltyramine per g of heart tissue, compared with previously reported concentrations of 2·8 and 2·4 nmole respectively of the amines 6 hr after treatment (Andén, 1964).

We have also used the method to separate the α-methylated amines excreted in the urine. The procedure used is as follows: 10 ml of urine is adjusted to pH 9 with sodium hydroxide and then shaken with 30 ml of 1:1 mixture of n-butanol and heptane for 6 min. After centrifugation, 25 ml of the organic phase is shaken with 10 ml of 0·01N HCl for 2 min. 5 ml of the acid phase is adjusted to pH 6·5 and passed through the Dowex resin. After elutions with N and 2N HCl, as previously described, metaraminol and methyltyramine are condensed with *o*-phthalaldehyde and their fluorescence read.

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The effect of theophylline on chloride permeability and active chloride transport in various epithelia

SIR,—Antidiuretic hormone (ADH) and theophylline are known to stimulate sodium transport across frog skin in the absence of electrical, chemical or osmotic gradients (Baba, Smith & Townshend, 1967). Fig. 1 shows the increase

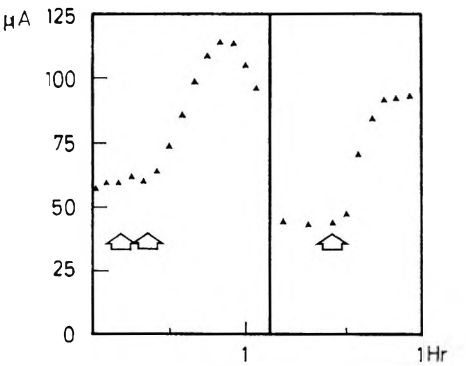

FIG. 1. Short circuit current measurements in 4 cm^2 pieces of frog abdominal skin (*R. temporaria*) bathed on both sides by frog Ringer. Left. At the arrows 130 and 260 mU/ml of arginine vasopressin were added to the solution bathing the inside of the skin. Right. At the arrow 1×10^{-3}M theophylline was added to the inner bathing solution.

in short circuit current (a measure of sodium transport [Ussing & Zerahn, 1951]) across abdominal skin of *Rana temporaria* caused by ADH and theophylline. The similarity of these responses has led to the idea for both frog skin (Baba and others, 1967) and other transporting epithelia (Orloff & Handler, 1967) that these two substances act through a final common pathway. The suggested common mediator is cyclic-3′,5′-AMP (Baba & others, 1967; Orloff & Handler, 1967) which, it is thought, increases the permeability of the outer facing membranes of the cells to sodium ions, thus supplying the actively pumping sites at an increased rate. It is proposed that endogenous levels of cyclic-3′,5′-AMP

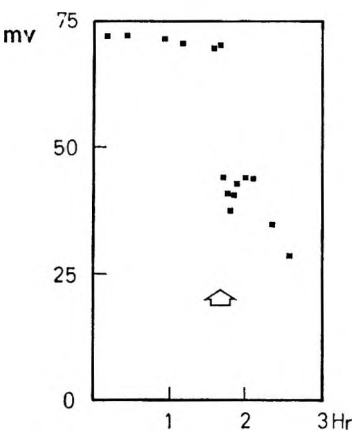

FIG. 2. The transepithelial potential of frog skin (*R. temporaria*) bathed in normal Ringer solution. At the arrow 1×10^{-2}M theophylline was added to the inner bathing solution.

are raised by activation of adenyl cyclase by ADH, or by prevention of destruction of the cyclic nucleotide by inhibiting phosphodiesterase with theophylline.

However, ADH and theophylline affect the frog skin potential in opposite ways. ADH causes an increase in the transepithelial potential, whereas the transepithelial potential falls on treatment of skins with theophylline. Fig. 2 shows the effect of theophylline on the skin potential of abdominal skin from *R. temporaria*. There is an immediate, precipitous fall followed by a somewhat slower fall. This effect of theophylline was dose dependent over the range 10^{-2} to 10^{-4}M. The increase in skin potential caused by ADH is understandable in terms of an increased permselectivity of the outer facing membranes to sodium ions (Civan, Kedem & Leaf, 1966), causing the outer surface to become more negative with respect to the inside. It was shown in further experiments that the fall in skin potential caused by theophylline did not depend on the presence of either sodium or potassium ions in the bathing fluid. The response did depend on a high concentration of chloride ions in the external medium, for instance when the skin was bathed in iso-osmotic choline chloride. It was also shown that the fall in skin potential resulted from changes occurring in the outer facing membranes of the skin even though the theophylline was applied to the inside of the skin. This was detected by using the technique described by Steinbach (1933). It is clear that if theophylline increases the permeability of the outer facing membranes to chloride ions then the movement of these ions down their chemical gradient generates a potential across the membrane such that the outer surface of the skin is less negative, thus the skin potential is reduced or may even be reversed.

The increase in sodium transport across the skin caused by theophylline results not from alterations in permeability of the membranes to sodium ions, but to chloride ions. In the open-circuited condition chloride ions exert an anion-drag on the movement of the actively transported species (Leaf, 1965). Even under short-circuited conditions anion-drag is still present, although to a lesser extent, since local potential gradients must still exist in a membrane with a mosaic of sodium and chloride permselective sites.

The actions of theophylline have been further investigated in two other actively transporting epithelia where active transport of chloride has been demonstrated. When the toad bladder (*Bufo marinus*) is bathed on both sides by Ringer solution deficient in potassium ions the transmembrane potential (serosal side positive) falls to zero and eventually reverses. Under these conditions a negative short circuit current is required to reduce the skin potential to zero.

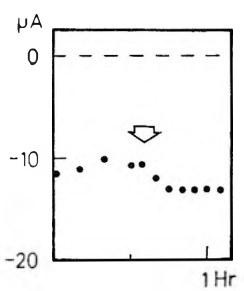

FIG. 3. Negative short circuit current measurements for 4 cm² of toad bladder (*B. marinus*). The bladder was bathed in potassium-free frog Ringer gassed with a mixture of 95% O_2 - 5% CO_2. At the arrow theophylline (1×10^{-3}M) was added to the inner bathing solution.

The negative short circuit current is equivalent to the mucosal to serosal active transport of chloride (Finn, Handler & Orloff, 1967). Theophylline applied to the serosal side of the bladder increased the extent of the negative short circuit current, as shown in Fig. 3.

Removal of sodium ions and reduction of chloride concentrations to low levels (2 m-equiv.) in the solution bathing the outside surface of the skin of *R. pipiens* abolishes sodium transport and exposes chloride transport (Martin, 1964; Martin & Curran, 1966). As in the toad bladder, active chloride transport was associated with a reversed potential and a negative short circuit current. In this case too, theophylline caused an increase in the negative short circuit current as shown in Fig. 4. The effects of theophylline on chloride transport

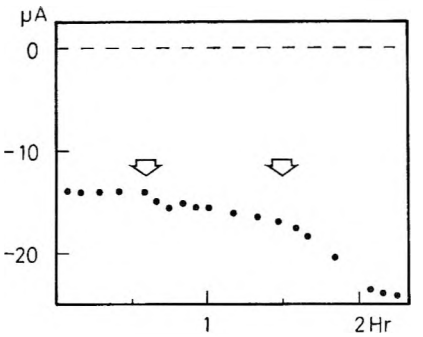

FIG. 4. Negative short circuit current measurements for 4 cm^2 frog skin (*R. pipiens*). The outer bathing solution was 2 mM potassium chloride, and the inner bathing solution was 2 mM potassium chloride and 56 mM sodium sulphate. Both solutions were made iso-osmotic with frog Ringer by addition of sucrose. At the first arrow 1×10^{-4}M theophylline and at the second arrow 1×10^{-3}M theophylline was added to the inner bathing solution.

are weak but chloride transport itself is not particularly marked. In the skin of *R. pipiens* chloride transport is only 5% of the sodium transport (Martin & Curran, 1966), while in the toad bladder sodium and chloride transport are mutually exclusive and it is suggested that the sodium and chloride ions use the same transfer system (Finn & others, 1967). The effects of theophylline on skin potential are, by comparison, dramatic. Perhaps the rate-limiting process in chloride transport is the chloride pumping, rather than access of chloride ions to the pumping sites. This differs from sodium transport across the epithelia where, quite clearly, the entry of sodium ions to the pumping sites limits the rate of sodium transport. Alternatively, chloride pumping in *R. pipiens* in the absence of external sodium ions may be restricted by cation drag.

The effects of theophylline on skin potential and negative short circuit current under the conditions described are consistent with the view that this agent increases the chloride permeability of the outer facing membranes. It also seems likely that the effect of theophylline on sodium transport in these various epithelia is due to the removal of anion-drag normally exerted on the sodium cation.

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Adrenergic receptors in the ruminal wall of sheep

SIR,—Ruminants suffering from infectious diseases often show a reduction in rumen motility, especially during the fever period. This effect can also be observed after injection of infusion fluids contaminated with pyrogens, or purified lipopolysaccharide from Gram-negative bacteria (Miert, 1966). We considered the possibility that the reduction in rumen motility after an injection with lipopolysaccharide is the result of sympathetic stimulation or adrenaline release (Miert, 1968). This led to a study of the adrenergic receptor in the rumen.

We know from the literature (summarized by Habel, 1956), that in the unanaesthetized ruminant with intact vagi, adrenaline inhibits rumen motility. In unanaesthetized vagotomized sheep it caused a single slow contraction of reticulum, rumen and abomasum. An intravenous injection of adrenaline in the anaesthetized goat results also in a contraction of the rumen.

Adrenaline on isolated strips of ruminal wall inhibited or stimulated the contractions (Dussardier & Navarro, 1953; Sanford, 1958). Duncan (1954) noted that the most characteristic effect of adrenaline on strips from the rumen abomasum and omasum of sheep was brief inhibition followed by contractions. With low doses, inhibition was the main effect, with high doses only strong contractions were observed. Dussardier & Navarro (1953) gave particular attention to the effects of adrenaline on strips of the abomasum. They noted that contractions from adrenaline could not be suppressed by atropine in concentrations which were sufficiently high to antagonize the action of acetylcholine. The adrenergic blocking agent 883F [2-(diethylaminomethyl)-1,4-benzodioxan], however, inhibited the motor effect of adrenaline.

For our experiments, strips of 7 × 2 cm were taken from the dorsal ruminal sac of sheep, immediately after slaughtering. These were transported in cooled Tyrode solution. In the laboratory, the serosa and mucosa layers were removed and the muscular layer placed in a bath with 50 ml of Tyrode solution without glucose, at 37° and aerated with an oxygen 95% and carbon dioxide 5% mixture. Recordings of the contractions were made isotonically on a kymograph (Stücklin, 1951). For specific α-receptor stimulation we chose oxymetazoline hydrochloride (Mujic & Rossum, 1965; Rossum & Mujic, 1965), and for specific β-stimulation we used isoprenaline hydrochloride, both at a concentration in the bath fluid of 0·2 μg/ml. Other agents were adrenaline hydrochloride (0·2 μg/ml), dibenamine hydrochloride (2 μg/ml), pronethalol hydrochloride (8 μg/ml) and Du 21445 [1-isopropyl-amino-3-(2-methylthiophenoxy)-propanol-2], also a strong β-blocking agent (2–4 μg/ml). The time intervals between the drug administration was usually about 30–40 min. After each response the bath fluid was renewed several times.

Results. Only about 10% of all strips showed spontaneous activity after a short incubation time, which subsided in a few hours as the experiment progressed. The activity usually consisted of monophasic contractions occurring 8 to 10 times/min.

Isoprenaline always gave a reduction in tension and very often a decrease of amplitude. This effect could be blocked by pronethalol, but not by dibenamine. Oxymetazoline caused always a sharp rise in tone, sometimes with a reduction of the amplitude. This effect could be blocked by dibenamine, but not by pronethalol. Some strips reacted with a strong contraction, others with a relaxation after exposure to adrenaline. The rise in tone could be blocked by dibenamine; thereafter, a second dose of adrenaline gave a relaxation, which in turn could be blocked by pronethalol. Strips which reacted first with a relaxation, gave a contraction to a second dose of adrenaline after pronethalol. Dibenamine added next, blocked this type of reaction. After Du 21445, oxymetazoline still gave a contraction, while the effect of isoprenaline was completely inhibited.

From these results, we conclude, that in ruminal smooth muscle preparations, there exist α-stimulatory and β-inhibitory receptors.

It is of interest that in the gastrointestinal tract of most species, both α- and β-receptor stimulation results in inhibition of contractions ((Ahlquist & Levy, 1959; Bucknell & Whitney, 1964; Rossum & Mujic, 1965; Takagi, Osada & others, 1967). Some exceptions, where motor effects of adrenaline were observed, have been listed by Dussardier & Navarro (1953) and recently by Christensen & Daniel (1968). In the ruminal wall, apparently the stimulation of α-receptors characteristically gives contraction.

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Letters to the Editor

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