JOURNAL OF PHARMACY AND PHARMACOLOGY

VOLUME XV No. 8

AUGUST 1963

Published by Direction of the Council of

THE PHARMACEUTICAL SOCIETY OF GT. BRITAIN

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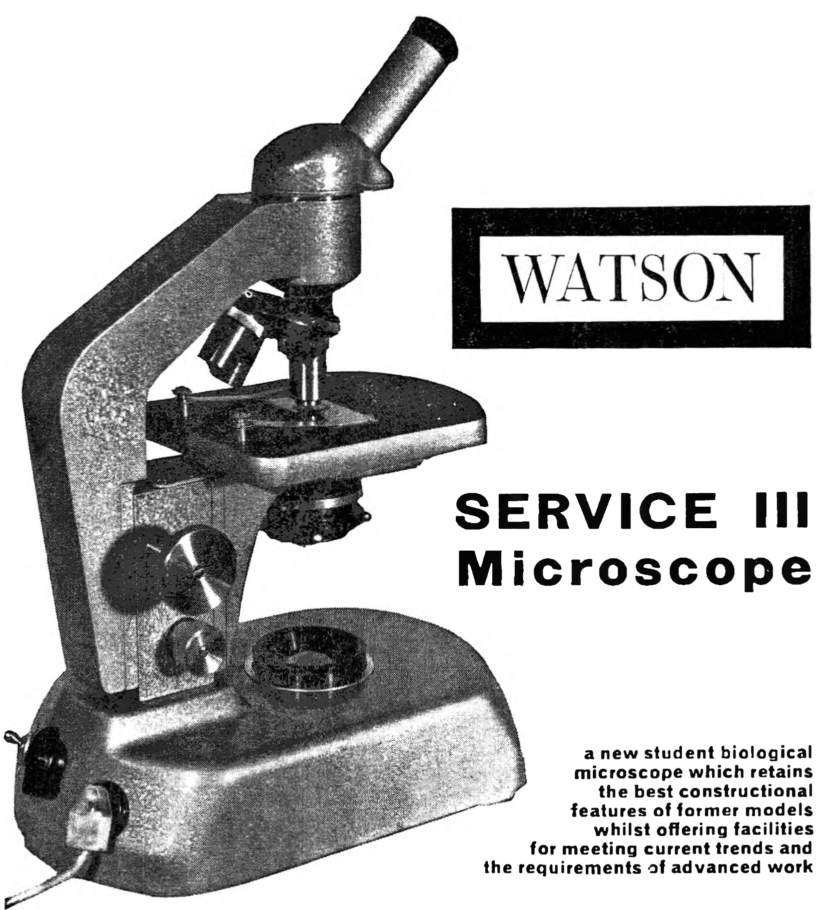

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RESEARCH PAPERS

REVERSAL OF THE EFFECT OF α-METHYLDOPA BY MONOAMINE OXIDASE INHIBITORS

By J. M. van Rossum and J. A. Th. M. Hurkmans

From the Department of Pharmacology, Medical School, University of Nijmegen, Nijmegen, Netherlands

Received May 31, 1963

L-α-Methyldopa, which normally causes sedation, induces a strong central excitation in mice pretreated with a monoamine oxidase inhibitor after a lag of a few hours. It is concluded that this excitation is caused by accumulation of free catecholamines liberated by amines which are slowly formed by decarboxylation of α-methyldopa. The hypotensive and sedative effects of α-methyldopa given alone are attributed to the slow release of catecholamines and subsequent breakdown by monoamine oxidase so that a partial depletion of catecholamines ensues.

Decarboxylase-inhibitors represent a new class of pharmacological agents (Sourkes, Murphy and Chavez-Lara, 1962). L-α-Methyl dioxyphenylalanine (α-methyldopa), which is a representative of this class, is widely used as an antihypertensive drug (Bayliss and Harvey-Smith, 1962; Dollery and Harington, 1962; Gillespie, Oates, Crout and Sjoerdsma, 1962; Kirkendall and Wilson, 1962; Oates, Gillespie, Udenfriend and Sjoerdsma, 1960). α-Methyldopa inhibits the enzymatic decarboxylation of L-dioxyphenylalanine (dopa) and of 5-hydroxytryptophan (5-HTP) (Clark, 1959; Sourkes, 1954).

The anti-decarboxylase properties of α-methyldopa have also been shown to exist *in vivo* both in animals (Clark, 1959; Dengler and Reichel, 1958; Hansson and Clark, 1962) and in man (Oates and others, 1961). Serious doubt has been expressed whether inhibition of dopa decarboxylase could be the cause of the antihypertensive properties of α-methyldopa (Gillespie and others, 1962; Hess, Connamacher, Ozaki and Udenfriend, 1961).

From enzymological studies it became evident that α-methyldopa is not only an inhibitor but also a substrate for dopa decarboxylase, having an affinity similar to dopa but a turnover-rate 200 times slower (Lovenberg, Weissbach and Udenfriend, 1962). Also *in vivo* α-methyldopa is slowly converted into the methyl-analogues of the catecholamines (Carlsson and Lindqvist, 1962). Furthermore, from clinical reports it appears that catecholamine-like substances are formed from α-methyldopa during therapy with this drug (Lauwers, Verstraete and Joossens, 1963; Stott, Robinson and Smith, 1963).

The metabolic products (2*S*-α-methyldopamine, 1*R*:2*S*-α-methylnoradrenaline*) formed from α-methyldopa are potent releasers of

**R* and *S* are notations of absolute configuration according to the sequence rule (Cahn, Ingold and Prelog, 1956).

endogenous catecholamines (Hess and others, 1961; Porter, Totaro and Leiby, 1961). Other far more potent decarboxylase inhibitors (see Table I) than α-methyldopa, themselves unaffected by dopa decarboxylase, have no catecholamine-releasing properties, do not cause depletion of catecholamines and have no antihypertensive effect (Brodie, Kuntzman, Hirsch and Costa, 1962; Drain, Horlington, Lazare and Poulter, 1962). It has recently been shown that these potent and pure decarboxylase inhibitors can completely abolish the catecholamine-depleting and hypotensive effects of α-methyldopa (Davis, Drain, Horlington, Lazare and Urbanska, 1963).

TABLE I

DOPA AND DECARBOXYLASE INHIBITORS

Affinity for the enzyme and relative intrinsic turn-over rate (k_3) obtained from enzymological studies (Lovenberg, Weissenbach, and Udenfriend 1962) and *in vivo* studies (Hansson and Clark, 1962)

Drug	Code	Rel. k_3	Relative affinity	
			Substrate	Inhibitor
HO, HO-phenyl-C-C(COOH)-NH_2	DOPA	100	1·3	—
HO, HO-phenyl-C-C(COOH)(C)-NH_2	α-MeDOPA	0·5	1	1
HO, HO-phenyl-C-C(COOH)(C)-NH-NH_2	MK-485	0	—	45
HO-phenyl-C-N(C)-NH_2	NSD-1034	0	-	45

MK-485 is 2-Hydrazino-4-(3,4-dihydroxyphenyl)-2-methylbutyric acid
NSD-1034 is *N*(*m*-hydroxybenzyl)-*N*-methylhydrazine

The conclusion is thus reached that α-methyldopa acts indirectly by being slowly converted into catecholamine analogues which in turn cause release of catecholamines. Since the catecholamines so released are simultaneously metabolised by the enzyme monoamine oxidase a partial depletion ensues.

The antihypertensive action of α-methyldopa thus closely resembles the effect of reserpine. Also α-methyldopa causes some degree of sedation in animals and man (Bayliss and Harvey-Smith, 1962; Oates and others, 1960).

It may be anticipated that when given after pretreatment with a monoamine oxidase inhibitor α-methyldopa will cause an accumulation of free catecholamines and thus hypertension and central excitation.

METHODS

Central effects of α-methyldopa were studied in female mice of the R.Q. strain (an F_1 hybrid of R, Rhodes farm albino, and Q, Extreme dilute). Motor activity was continuously registered with cumulative recorders (Rossum, 1962). Mice of a homogeneous population were

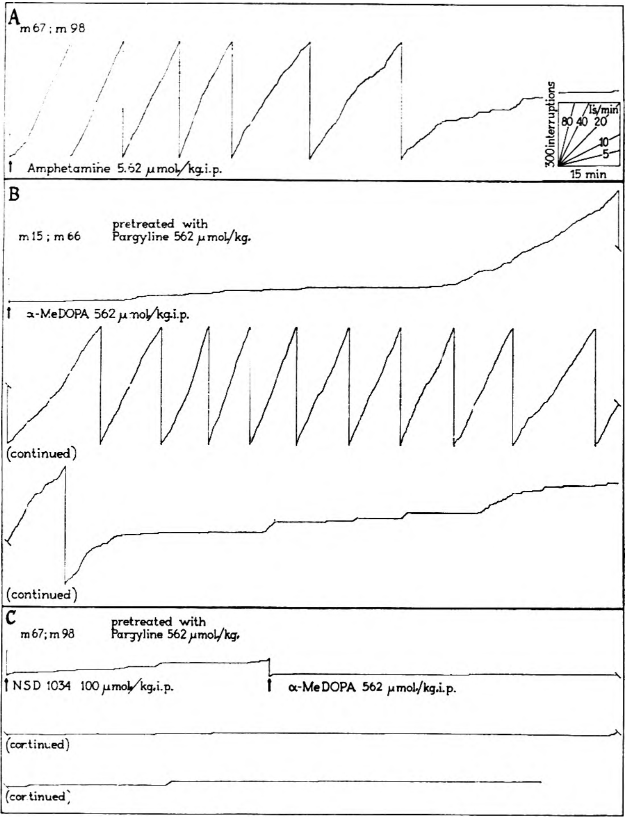

FIG. 1. Cumulative records of the motor activity of two mice under various experimental conditions. In A, mice Nos. 67 and 98 received dexamphetamine (1 mg./kg. of the sulphate) intraperitoneally. The onset of dexamphetamine is almost instantaneous. In B, mice Nos. 15 and 66 which 2 hr. earlier were injected with pargyline (119 mg./kg.) received α-methyldopa (118 mg./kg.). The pargyline as such does not cause an effect. After a lag period of more than 2 hr. a strong increase in locomotor activity occurs which lasts for hours. In C, mice Nos. 67 and 98 which first received pargyline followed 30 min. later by NSD-1034 were injected again 90 min. later with α-methyldopa (118 mg./kg.). The decarboxylase inhibitor completely abolished the central stimulant action of the combination of α-methyldopa and pargyline.

selected for similar sensitivity to dexamphetamine. All mice received a test dose of 5·62 μmol./kg. dexamphetamine (1·0 mg./kg. of the sulphate) one day before the experiment. (See upper row in Fig. 1.) The experiments were conducted in three groups of two mice. A dose of 562 μmol./kg. α-methyldopa (119 mg./kg.) was injected i.p. into (*a*) mice

of the control groups; (*b*) mice which had received 562 μmol./kg. pargyline (*N*-benzyl-*N*-methyl-2-propynylamine hydrochloride) (113 mg./kg. of hydrochloride) 2 hr. previously; and (*c*) in mice that in addition to pargyline also received 100 μmol./kg. *N*-(*m*-hydroxybenzyl)-*N*-methyl-hydrazine (NSD-1034) (25 mg./kg. of H_2PO_4 salt) 30 min. before the α-methyldopa. A typical experiment is presented in Fig. 1. Other groups of two mice were reserpinised by administration of 1 mg./kg.

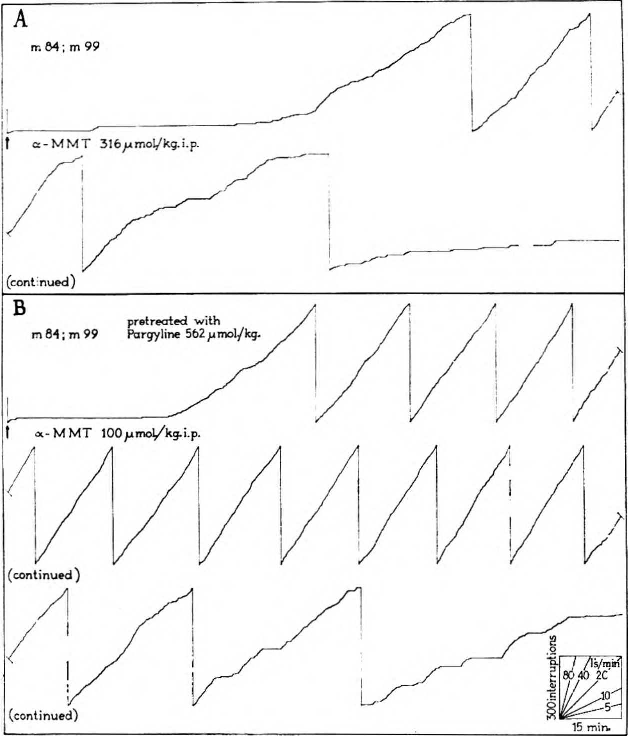

FIG. 2. Cumulative records of motor activity in mice under the influence of α-MMT. In A, mice Nos. 84 and 99 were injected with α-MMT (60 mg./kg.) intraperitoneally. After a lag period of less than 1 hr. an increase of locomotor activity occurs. In B, the same mice one day later were first injected with pargyline (119 mg./kg.) followed 2 hr. later by a threefold smaller dose of α-MMT (19·5 mg./kg.). After a lag time of about 45 min. a strong increase in locomotor activity occurs. The psychomotor stimulant action of α-MMT is potentiated by a monoamine oxidase inhibitor by more than a factor five.

reserpine i.p. over 3 or 4 subsequent days, after which they received 562 μmol./kg. pargyline, followed 2 hr. later by 562 μmol./kg. α-methyldopa. The analogous amino-acid DL-α-methyl-*m*-tyrosine (α-MMT) was also studied in groups of two mice alone in doses of 316 and 562 μmol./kg. and 2 hr. after 562 μmol./kg. pargyline in doses of 31·6 and 100 μmol./kg. A typical experiment is given in Fig. 2.

Results and Discussion

It was observed that α-methyldopa alone produces a decrease in locomotor activity. In mice pretreated with a monoamine oxidase inhibitor (pargyline) administration of α-methyldopa caused a strong and prolonged increase of motor activity and central excitation resembling an overdose of dexamphetamine. This reversed effect of α-methyldopa occurred after a lag of 2 to 3 hr. (Fig. 1). In contrast, the central stimulant action of dexamphetamine is almost instantaneous. These experiments suggest that the lag in time is caused by a slow conversion in the brain of α-methyldopa to α-methyldopamine or α-methylnoradrenaline or both. This view was further substantiated by experiments with mice which, in addition to a monoamine oxidase inhibitor, also received a strong decarboxylase inhibitor (NSD–1034). The latter drug abolishes the central stimulant action of α-methyldopa in mice pretreated with pargyline. It may be noted here that the catecholamine-depleting and the hypotensive effects of α-methyldopa alone also occur after a lag period of a few hours and that these actions are also completely abolished by the inhibition of dopa decarboxylase (Davis and others, 1963; Drain and others, 1962) suggesting a common mechanism of action.

From the experiments shown in Fig. 1 it seems likely that a release of catecholamines by the amines which are slowly formed from α-methyldopa is the cause of the central stimulant action of the combination of α-methyldopa with the monoamine oxidase. But separately or together it seems unlikely that α-methyldopamine and α-methylnoradrenaline are themselves responsible for the central stimulant action of the combination. In that case it would be expected that α-methyldopa alone would produce a central stimulant action, since α-methyl analogues are resistant to monoamine oxidase. On the contrary, the experiments provide evidence that the α-methyl catecholamines formed from α-methyldopa cause a release of endogenous catecholamines, the oxidation of which is prevented by a monoamine oxidase inhibitor. Further evidence for this supposition is gained by experiments in which the combination of α-methyldopa and pargyline was given to mice previously treated with reserpine. When the catecholamines have been depleted the combination does not exert central excitation. Furthermore, after a large dose of α-methyldopa (1,000 μmol./kg.) alone is given to mice which then receive monoamine oxidase inhibitor the next day, a subsequent dose of α-methyldopa is ineffective as a stimulant. Obviously therefore replenishment of catecholamine stores is essential for the central stimulant action of α-methyldopa when given after a monoamine oxidase inhibitor.

The experiments lend no support to the recent postulation that α-methyldopa could act as a precursor of a false transmitter of noradrenaline (Day and Rand, 1963). Also results from the biochemical work (Gessa, Costa, Kuntzman and Brodie, 1962) pleads against this supposition. α-Methyldopa as well as the analogous amino-acid DL-α-methyl-*m*-tyrosine (α-MMT) cause a depletion of noradrenaline which lasts several days, whereas methyltyramines can be detected only during the first 24 hr. after administration (Carlsson and Lindqvist, 1962).

α-MMT acts similarly by virtue of its decarboxylation products which are potent releasers of catecholamines (Porter and others, 1961; Udenfriend and Zaltman-Nirenberg, 1962). α-MMT causes a central excitation of its own after a lag of about 45 min. (Rossum 1963a). Thus it is a better substrate for dopa decarboxylase than α-methyldopa while the amines formed from it are better releasers of catecholamines. The central stimulant action of α-MMT is strongly potentiated by monoamine oxidase inhibitors (see Fig. 2). Its stimulant action is also dependent on replete catecholamine stores (Rossum, 1963a; Gessa and others, 1962).

The antihypertensive action of α-methyldopa seems unique for this amino-acid, since it is a substrate for dopa decarboxylase with such a low turnover rate that the amines formed from it cause in turn a slow release of endogenous catecholamines. As a result, breakdown by monoamine oxidase keeps up with the release so that depletion of catecholamines occurs without induction of central excitation. Therapeutically α-MMT is inferior because its decarboxylation products cause too fast a release of catecholamines.

A consequence of the mechanism of action of α-methyldopa is its reversal of action by monoamine oxidase inhibition. It might therefore be dangerous to begin therapy with α-methyldopa in patients who have been treated with a monoamine oxidase inhibitor in the two preceding weeks (Rossum, 1963b), whereas administration of monoamine oxidase inhibitors during α-methyldopa therapy is thought to be less dangerous.

Acknowledgements. We are grateful to Dr. C. A. Stone, Merck, Sharpe and Dohme, Westpoint, Pa., for the generous donation of L-α-methyldopa (Aldomet) and DL-α-methyl-*m*-tyrosine; to Dr. G. M. Everett, Abbott Laboratories, North Chicago, Ill., for pargyline HCl, and to Dr. D. J. Drain, Smith and Nephew Research Ltd., Ware, Herts., for NSD 1034 phosphate.

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THE FATE OF TRITIUM-LABELLED β-GLYCYRRHETIC ACID IN THE RAT

By D. V. Parke, Sylvia Pollock and R. T. Williams

From the Department of Biochemistry, St. Mary's Hospital Medical School, London, W.2

Received March 15, 1963

Tritium-labelled β-glycyrrhetic acid was prepared and administered orally, subdermally and intraperitoneally to male and female rats. Most of the radioactivity was excreted in the faeces, only traces being found in the urine. Administered to rats with biliary cannulae most of the radioactivity was excreted in the bile. Three metabolites of β-glycyrrhetic acid have been detected in the bile, but these have not yet been identified.

β-Glycyrrhetic acid (3-hydroxy-11-oxo-18-β-olean-12-en-30-oic acid) is a triterpenoid which occurs as the diglucuronide, glycyrrhizic acid, in liquorice root. β-Glycyrrhetic acid and its derivatives have been used in the treatment of gastric ulcer (Doll, Hill, Hutton and Underwood, 1962) and of dermatitis (Colin-Jones, 1960) and it has been shown to reduce hypercholesteraemia (Shibata, 1961). The metabolism of orally administered tritium-labelled glycyrrhetic acid and monoammonium glycyrrhizate in man has been studied by Carlat, Margraf, Weathers and Weichselbaum (1959), who suggested that both these compounds were poorly absorbed from the gastrointestinal tract, since most of the material was excreted in the faeces. We have now studied the fate of tritium-labelled β-glycyrrhetic acid in rats and have found that the compound is absorbed well from the alimentary canal. Most of the radioactive material is excreted in the bile and is then eliminated in the faeces, mainly as metabolites.

β-Glycyrrhetic Acid

Experimental

Materials

β-Glycyrrhetic acid randomly labelled with tritium was prepared from tritiated water and unlabelled β-glycyrrhetic acid, m.p. 284 − 7°, $[\alpha]_D^{19}$ + 163° ± 1° (*c*, 1 in $CHCl_3$) by the method of catalytic exchange (see Gould, 1958). 3-Acetyl-β-glycyrrhetic acid (1·2 g.) together with 660 mg. of tritiated water (2·5 c, Radiochemical Centre, Amersham), 3·3 ml.

acetic acid and 67 mg. of pre-reduced platinum oxide catalyst (Adams, Voorhees and Shriner, 1941) were heated at 127° for 72 hr. in a sealed tube. The product, dissolved in 5 ml. water and 135 ml. of a 2·5 per cent w/v solution of potassium hydroxide in methanol, was then hydrolysed by refluxing under nitrogen for 2 hr. The hydrolysed solution was acidified with 100 ml. 2N hydrochloric acid and the white precipitate of tritium-labelled β-glycyrrhetic acid which formed was extracted into 150 ml. chloroform. The aqueous layer was further extracted with two 50 ml. portions of chloroform and the combined extracts washed twice with 100 ml. water, dried over anhydrous sodium sulphate and the solvent evaporated. The crude product (yield, 1·07 g., m.p. 270–275°) was recrystallised from a mixture of methanol and chloroform to give pure tritium-labelled β-glycyrrhetic acid (0·8 g.), m.p. 281–5° $[\alpha]_D^{22} + 169° \pm 2°$ (*c*, 1 in $CHCl_3$), 3 mc/g. The product gave a single spot when run on a paper chromatogram by descending chromatography on Whatman No. 1 paper in a solvent consisting of light petroleum (b.p. 80–100°): methanol: water (5:4:1 by vol.) (Bush, 1952). The infra-red spectra was identical with that of authentic β-glycyrrhetic acid.

Methods

Animal experiments. ^{3}H-β-Glycyrrhetic acid was administered orally to albino rats (weighing *ca.* 150 g.) as an aqueous suspension or as a solution in arachis oil, and intraperitoneally as a solution in propylene glycol.

Determination of radioactivity. In preliminary experiments, the faeces, urines and tissues were dissolved in formamide (Kinnory, Kanabrocki, Greco, Veatch, Kaplan and Oester, 1958) and the radioactivity counted in a Panax refrigerated scintillation counter type SC–LP at 7°. A solution of a mixture of naphthalene, 2,5-diphenyloxazole (PPO), 1,4-bis-[2′-(5′-phenyloxazole)]-benzene (POPOP) (800:50:1, by weight) in toluene: ethanol:dioxane (5:4:3, by volume) (TED/5 Panax) was used as phosphor. In subsequent experiments, tissues, faeces, urine and bile were dried in cellophane packets under infra-red lamps, and then ignited in an atmosphere of oxygen (Kelly, Peets, Gordon and Buyske, 1961). The ^3H_2O formed by the combustion was dissolved in TED/5 phosphor (Panax) and the solution counted as before. Results obtained by these two different methods showed no significant differences, but the latter method eliminated the high tissue blanks observed in the formamide method.

Biliary cannulation. To investigate the possibility of biliary excretion of glycyrrhetic acid or its metabolites, ^{3}H-β-glycyrrhetic acid was administered orally or intraperitoneally to albino rats after surgical insertion of a polythene cannula into the common bile duct (Stewart and Harrison, 1961). The rats were kept in wire restraining cages (Bollman, 1948) and were allowed free access to an aqueous solution of glucose (5 per cent w/v) -saline (1 per cent w/v), with or without addition of bile salt (0·04 per cent w/v). The bile, urine and faeces were collected at intervals over a period of three days and together with tissues, were combusted to ^3H_2O and the radioactivity measured.

Chromatography of metabolites. The bile obtained from cannulated rats dosed with ^{3}H-β-glycyrrhetic acid was first chromatographed on Whatman No. 1 paper in solvent A, i.e. chloroform: acetic acid: water (2:1:1 by vol.). Radioactive material remaining at the origin was eluted and further chromatographed on Whatman 3 MM paper in solvent B, i.e. the lower phase of 70 per cent v/v aqueous acetic acid: 1,2-dichloroethane: n-butanol (10:9:1 by vol.) by the descending technique (Sjovall, 1955). The chromatograms were cut into strips and the positions of the radioactive metabolites determined by absorption in ultra-violet light and by combusting 1 cm. bands of the paper and counting the $^{3}H_2O$ produced.

Estimation of unchanged β-glycyrrhetic acid in faeces. The unchanged β-glycyrrhetic acid in the rat faeces was determined by a reverse isotope dilution procedure. β-Glycyrrhetic acid (250 mg.) was added to dried faeces (1·0 g.) and the mixture extracted with chloroform in a Soxhlet apparatus for 1·5 hr. The chloroform extract was concentrated to 5 ml. and an aliquot (one-fifth) was chromatographed on Whatman 3 MM paper in solvent A. The area of the chromatogram between R_F 0·6–1·0 was cut out and eluted with chloroform: methanol (9:1 by vol.) and the eluate re-chromatographed on Whatman No. 1 paper in light petroleum (b.p. 80–100°): methanol: water (5:4:1) (solvent C). The area between R_F 0·0–0·17 of the second chromatogram was cut out and eluted as before and the eluate evaporated to dryness (52 mg.). An aliquot (ca. 1 mg.) of the residue was ignited and the radioactivity of the water of combustion determined.

TABLE I

PAPER CHROMATOGRAPHY OF THE GLYCYRRHETIC ACIDS AND SOME DERIVATIVES

Chromatograms were run on Whatman No. 1 paper in solvent A: chloroform: acetic acid: water (2:1:1 by volume); B: the lower phase of 70 per cent v/v aqueous acetic acid: 1,2-dichloroethane: n-butanol (10:9:1) (Sjovall, 1956); and C: light petroleum (b.p. 80–100°): methanol: water (5:4:1, by volume) by descending technique (Bush, 1952).

Compound	R_F value in solvent		
	A	B	C
β-Glycyrrhetic acid	0·98	0·95	0·1
α-Glycyrrhetic acid	1·0	1·0	0·0
3-Keto-β-glycyrrhetic acid	1·0	1·0	0·0
3-Acetyl-β-glycyrrhetic acid	1·0	1·0	0·57
Ammonium glycyrrhizinate	0·06	0·60	0·0
Metabolite I	0·0	0·40	0·0
Metabolite II	0·0	0·46	0·0
Metabolite III	0·44	0·77	0·0

Chromatography in solvent A separates β-glycyrrhetic acid from its metabolites, which are found at the origin and at R_F 0·1–0·4 (see Table I) and chromatography in solvent C separates β-glycyrrhetic acid from less polar compounds naturally present in faeces.

RESULTS

After administration of an oral dose of 60 mg./kg. of finely ground ^{3}H-β-glycyrrhetic acid suspended in water (3 ml.) to female rats, an

average of 86 per cent of the administered radioactivity was recovered in 1–3 days, 83 per cent being found in the faeces, 4 per cent in the liver and 1 per cent in the urine (see Table II). After sub-dermal injection of an aqueous suspension of ^{3}H-β-glycyrrhetic acid (60 mg./kg.) to female rats, an average of 74 per cent of the administered radioactivity was recovered in 5 days, 73 per cent being in the faeces and 1 per cent in the urine. Of the radioactivity present in the faeces, only 7·4 per cent was found to be β-glycyrrhetic acid when the triterpenoid was administered orally, and only 5·2 per cent when it was administered sub-dermally (see Table II). The levels of radioactivity found in the liver and gastrointestinal contents after oral administration were found to vary with the duration of the experiment in a manner suggestive of an entero-hepatic circulation of β-glycyrrhetic acid.

TABLE II

FATE OF ^{3}H-β-GLYCYRRHETIC ACID IN RATS

Expt. No.	Sex	Duration of expt. (days)	Dose per cent of radioactivity found in			Total per cent of dose accounted for
			Faeces	Urine	Liver	
Oral doses of 60 mg./kg. in aqueous suspension:						
1	F	1	70*	—	7·5	78
2	F	1·5	112*	—	2·6	115
3	F	2	82**	1·0	—	83
4	F	3	56	1·2	—	57
5	F	3	94	—	3	100†
6	M	2	46*	—	9·3	56
Sub-dermal doses of 60 mg./kg. in aqueous suspension:						
7	F	5	84‡	0·8	—	85
8	F	5	62	1·2	—	63

* Also includes gastrointestinal contents.
† Includes 3 per cent found in gastrointestinal contents.
** 7·4 per cent present as β-glycyrrhetic acid.
‡ 5·2 per cent present as β-glycyrrhetic acid.

Biliary excretion. After oral administration of ^{3}H-β-glycyrrhetic acid as a solution in arachis oil at a dose level of 25 mg./kg. to female rats, more than 70 per cent of the dose of radioactivity was excreted in the bile in 2–3 days, 13–20 per cent was found in the faeces and 2–3 per cent in the urine (see Table III). After oral administration of aqueous suspensions (25 mg./kg.) to male rats an average of 53 per cent of the dose of radioactivity was excreted in the bile in 3 days, a further 11 per cent was excreted in the faeces and 2 per cent in the urine. Female rats in the same time excreted 54 per cent in the bile, 15 per cent in the faeces and 9 per cent in the urine.

The biliary excretion was much greater and much more rapid after intraperitoneal administration of a solution of ^{3}H-β-glycyrrhetic acid in propylene glycol (see Fig. 1). At a dose level of 25 mg./kg., both male and female rats excreted an average of 100 per cent of the administered radioactivity within 12 hr. In fact, 95 per cent of the dose was excreted within 6 to 8 hr. in both sexes. The addition of 0·04 per cent w/v of ox bile salt to the liquid diet did not appear to alter significantly the total

biliary excretion or absorption of the triterpenoid, although in some experiments the initial rate of biliary excretion is slightly lower when bile salt is present in the diet.

Biliary metabolites of β-glycyrrhetic acid. The pooled bile obtained from three female rats, each of which had received 4 mg. of ^{3}H-β-glycyrrhetic acid in propylene glycol intraperitoneally, was chromatographed on Whatman No. 1 paper in solvent A. Two radioactive areas were detected with R_F values of 0–0·1 and 0·15–0·30.

TABLE III

FATE OF ^{3}H-β-GLYCYRRHETIC ACID IN RATS AFTER BILIARY CANNULATION

Expt. No.	Sex	Duration of expt. (days)	Dose per cent of radioactivity found in			Alimentary canal and contents	Total accounted for
			Faeces	Bile	Urine		
Oral doses of 25 mg./kg. in arachis oil:							
9	F	1·5*	7	36	2·4	—	46*
10	F	2	13	72	2·7	—	88
11	F	3	20	73	1·6	—	95
Oral doses of 25 mg./kg. as aqueous suspension:							
12†	F	2·5	14	56	6·6	1·1	77
13	F	3	17	51	12	0·3	81
14†	F	3	31	27	1·1	—	60**
15	M	3	5	55	1·6	0·3	62
16†	M	3	17	56	—	—	73
17†	M	3	—	48	—	—	48
Intraperitoneal doses of 25 mg./kg. in propylene glycol:							
18	F	2	—	93	—	—	93
19	F	2	—	102	—	—	102
20	F	2	—	105	—	—	105
21	M	2	—	102	—	—	102
22	M	2	—	83	—	—	83
23†	M	2	—	109	—	—	109

* Animal died before completion of expt.
** Flow rate of bile impeded, 0·6 per cent in the liver.
† Bile salt included in diet.

The material of R_F 0–0·1 was eluted with a mixture of methanol-chloroform (9 : 1 by volume) and the eluate was found to contain radioactivity equivalent to 4·5 mg. of the administered ^{3}H-β-glycyrrhetic acid or to 38 per cent of the dose. Further chromatography of this material on Whatman 3 MM paper in solvent B by descending chromatography revealed two ultra-violet-absorbent, radioactive spots with R_F values of 0·40 (metabolite I) and 0·46 (metabolite II). These two substances were separated by allowing the solvent to run off the front of the paper, the chromatograms being run for a period of 31 hr. The two spots were cut out, eluted with methanol : chloroform (9 : 1 by volume) and the solvent evaporated to give a semi-crystalline residue in each case (24·6 mg. containing metabolite I and 27·9 mg. containing metabolite II).

The material of R_F 0·15–0·30 in solvent A was similarly eluted with a mixture of methanol : chloroform (1 : 1) and the eluate was found to contain radioactivity equivalent to 4·3 mg. of the administered ^{3}H-β-glycyrrhetic acid or to 36 per cent of the dose. Evaporation of the solvent gave a semi-crystalline residue containing metabolite III (32·2 mg.).

The material containing metabolite I, but neither of the other two metabolite fractions, gave a strongly positive naphthoresorcinol reaction for glucuronic acid.

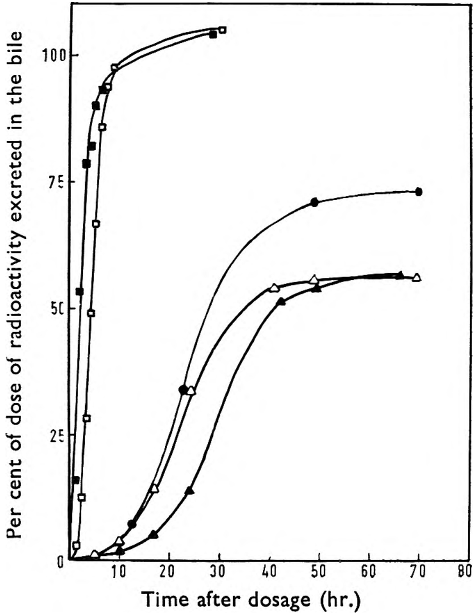

FIG 1. The rate of biliary excretion of radioactivity following administration of tritium labelled β-glycyrrhetic acid, intraperitoneally in propylene glycol to male rats, □, to female rats, ■; orally as an aqueous suspension to male rats, △, to female rats ▲; and orally as a solution in arachis oil to female rats, ●.

DISCUSSION

Tritium-labelled β-glycyrrhetic acid administered to rats is eliminated from the animal *via* the bile, for almost the whole of an intraperitoneal dose is excreted by this route and only traces are excreted in the urine. The extent and rate of the biliary excretion of this compound is approximately the same in both sexes.

When the compound is administered orally, the biliary excretion is less and the rate of excretion slower than when it is given intraperitoneally. Moreover, the biliary excretion is less and the rate of excretion slower when the oral dose is administered as an aqueous suspension than when it is administered as a solution in arachis oil. Both of these facts suggest that absorption from the gastrointestinal tract is slow, particularly when an aqueous suspension is fed. The replacement of the bile salt lost through cannulation by supplementation with ox bile salt in the diet has no marked effect.

In the previous experiments of Carlat, and others (1959) in which tritium-labelled β-glycyrrhetic acid was fed to man, most of the compound was recovered unchanged from the faeces, only trace amounts were found in the urine, and no activity was detected in the bile. This may have been

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because bile was obtained during only the first 4 hr. after dosage, and from our experiments with rats, one would expect the biliary excretion to be about one per cent of the dose, or less, during this period. We likewise found that in the normal rat without biliary fistula, most of the compound was recovered from the faeces, but only a small proportion of this was unchanged β-glycyrrhetic acid. Moreover, since the amount of β-glycyrrhetic acid present in the faeces after oral administration (7·4 per cent) was only slightly greater than that found after injection of the triterpenoid (5·2 per cent), the orally administered compound is probably almost completely absorbed.

The three products excreted in the bile have not as yet been identified but none is identical with unchanged β-glycyrrhetic acid. This may explain why β-glycyrrhetic acid has a healing action for gastric but not duodenal ulcers (see Doll and others, 1962). The β-glycyrrhetic acid, being a weak acid, should be largely absorbed from the stomach (Brodie and Hogben, 1957) and although the drug is almost completely excreted again into the duodenum *via* the bile, it is now present only as metabolites, which may have no healing activity.

Acknowledgement. This work was supported by a Grant from Biorex Laboratories Ltd., who also supplied us with glycyrrhetic acid and its derivatives.

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PHASE EQUILIBRIA IN SOME BETAINE-BENZALDEHYDE-WATER SYSTEMS

By J. Swarbrick and J. E. Carless

From the Chelsea School of Pharmacy, Chelsea College of Science and Technology, London, S.W.3

Received January 31, 1963

The phase equilibrium diagrams for six betaine-benzaldehyde-water systems have been determined at 25°, with particular reference to those phases in which future oxidation studies are to be carried out. A progressive increase in the amount of benzaldehyde solubilised in aqueous betaine solutions was found on increasing the betaine chain length from 8 to 11 carbon atoms. Further increase in the betaine chain length from 12 to 16 carbon atoms brought about a progressive decrease in benzaldehyde solubility. These results are discussed in the light of the phases separating at these solubility limits.

Work by Carless and Mitchell (1962) showed that the oxidation of aliphatic aldehydes dispersed in cetomacrogol solutions depended on the saturation of the dispersion and not on the concentration of the aldeyde and cetomacrogol, except in so far as these controlled the saturation. Recent work by the present authors (Carless and Swarbrick, 1962) on the oxidation of benzaldehyde emulsified and solubilised in aqueous solutions of betaines showed that the rate of oxidation did not, in the systems examined, depend upon the saturation ratio R, as defined by Carless and Mitchell. Since therefore the conception of R did not appear to be of a fundamental nature we expressed the feeling that, due to the likely complex nature of the dispersions, it was only from a consideration of the ternary phase diagrams for these systems that it might be possible to relate oxidation rates to the concentration and nature of the oil present.

In the furtherance of this hypothesis we have determined the relevant parts of the ternary phase diagrams for six different betaine-benzaldehyde-water systems, in which the alkyl chain length of the betaine molecule contained 8, 10, 11, 12, 14 and 16 carbon atoms.

Experimental

Materials Used

Benzaldehyde. Analar benzaldehyde was distilled under reduced pressure and stored in ampoules, under nitrogen, in the dark until required for use. N-*Alkyl* NN-*dimethylglycines* (*Betaines*). These ampholytic surface active agents, of general formula

$$R{-}\overset{+}{N}(Me)_2{-}CH_2COO^-$$

were prepared by the method of Beckett and Woodward (1963). Those homologues in which R, a normal alkyl chain, contained 8, 10, 11, 12,

14 and 16 carbon atoms were used. Distilled water was used in the preparation of all dispersions.

Determination of the Phase Boundaries

Dispersions containing two of the three components were prepared such that the percentage w/w of each was accurately known. All such two component dispersions lie on one of the three lines which form the boundary of the triangular diagram. A weighed amount of the third component was then added, dispersed by shaking and the whole system

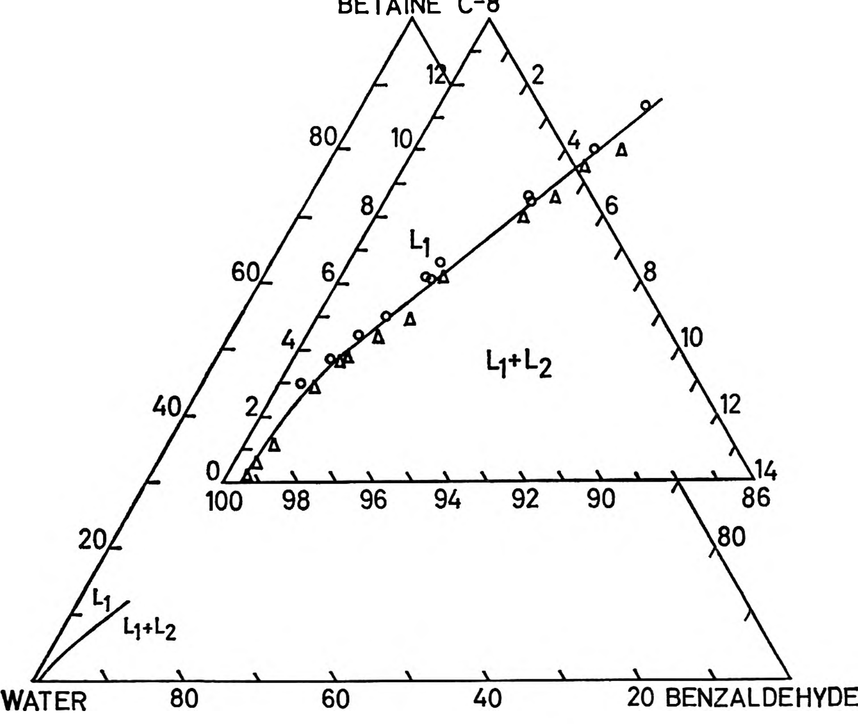

FIG. 1. System containing C_8 betaine.

○ Dispersions found to contain phase L_1.
△ Dispersions found to contain phases L_1 and L_2.

allowed to reach equilibrium at 25°. If necessary the system was centrifuged for 3 to 5 min. and re-equilibrated at 25°. The phases present were noted, the system redispersed, and centrifuged again. Only when the separated phases remained unchanged after being returned to the water-bath at 25° following centrifuging, was the system taken to be in equilibrium. The period of centrifuging was kept to a minimum since prolonged centrifuging causes an appreciable rise in temperature, thus altering the equilibrium of the system.

All dispersions were examined under polarised light, where a birefringent appearance denoted the presence of a liquid crystalline phase.

The addition of known amounts of the third component was continued until a change in the number of phases occurred. Calculation of the weight percentage of each component present enabled this phase change to be defined on the ternary phase diagram. Further known increments of the third component were then added and subsequent phase changes, if any, plotted on the triangular diagram.

The above procedure was repeated with different mixtures of betaine and water, water and benzaldehyde and benzaldehyde and betaine. Benzaldehyde, betaine and water were used respectively as the diluents.

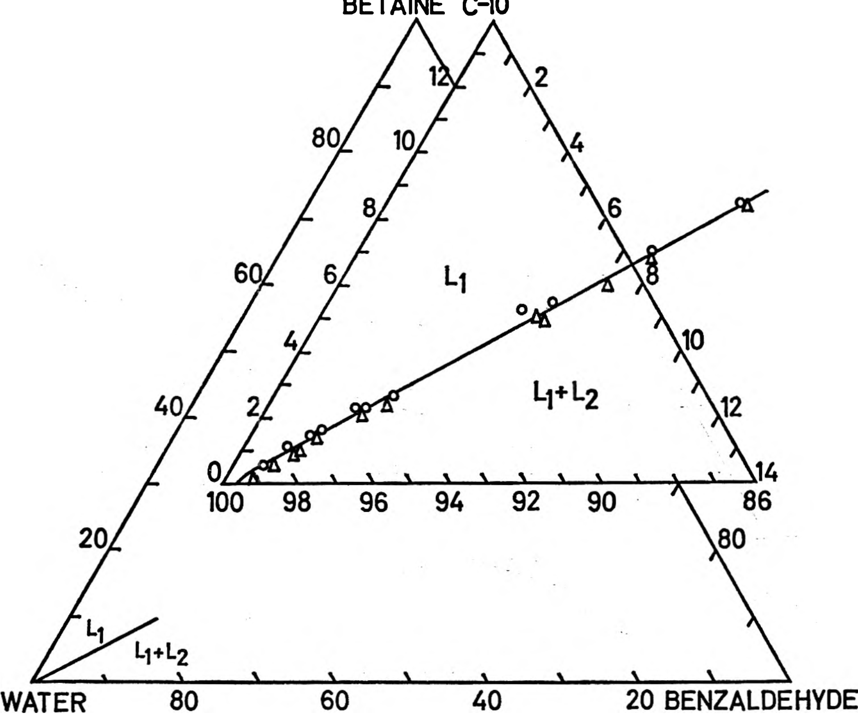

FIG. 2. System containing C_{10} betaine. Symbols as for Fig. 1.

The following terms are used in all subsequent discussions to depict the various phases found to exist in these systems. The region designated L_1 consists of isotropic liquid, the phase boundary being equivalent to the solubility curve for benzaldehyde in aqueous solutions of a particular betaine. L_2 describes a second isotropic liquid phase, the boundary in this case marking the limit of solutibility of water in benzaldehyde-betaine dispersions. The region $L_1 + L_2$ contains two isotropic liquid phases and is the only region where the conventionally defined emulsion is capable of existence. LC denotes an anisotropic liquid crystalline phase, or mesophase, which is intermediate in structure between the liquid and crystalline states. The molecules of the liquid crystalline

phase possess an ordered, laminar arrangement. The phase has a birefringent appearance under polarised light. The region $L_1 + LC$ contains both an isotropic liquid phase L_1 and a liquid crystalline phase LC. The triangular, ternary phase region contains all three phases and is therefore designated $L_1 + L_2 + LC$. Winsor (1954) has used the terms S_1, S_2 and G to describe the phases denoted here by L_1, L_2 and LC.

All systems are referred to by the length of the alkyl chain of the betaine homologue they contain. Thus the system betaine C_8-benzaldehyde-water is denoted by the term C_8.

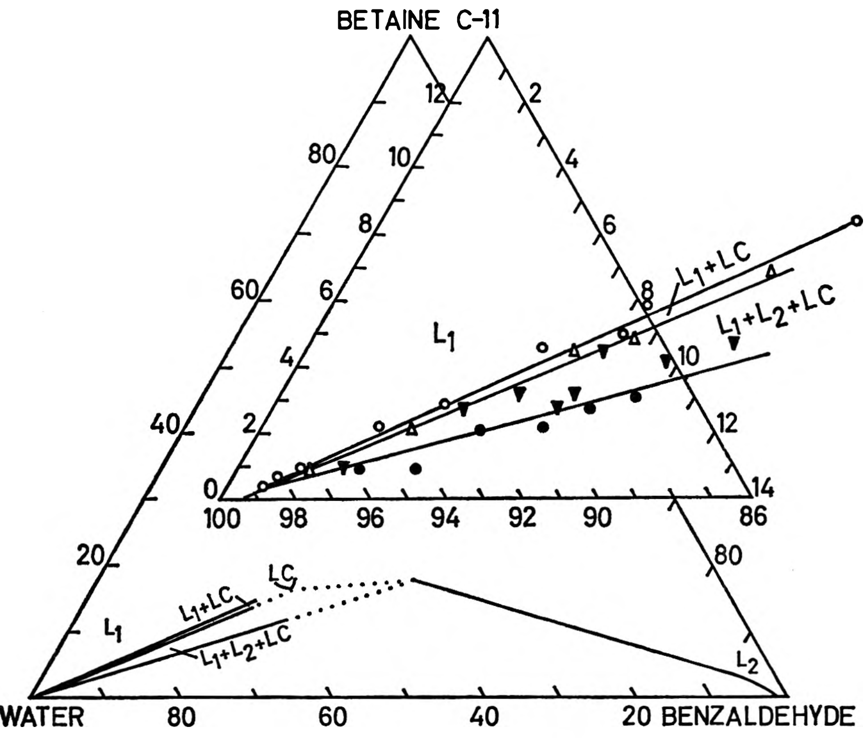

FIG. 3. System containing C_{11} betaine.
○ Dispersions found to contain phase L_1.
△ Dispersions found to contain phases L_1 and LC.
▼ Dispersions found to contain phases, L_1, L_2 and LC.
● Dispersions found to contain phases L_1 and L_2.

RESULTS

Since no quantitative determinations of the composition of the phases present were attempted in the present work there was no need to achieve complete separation of the phases. However a certain degree of separation was necessary in order to determine both the number and type of phase present. Normally, dispersions separate into their constituent phases if allowed to stand at the equilibrium temperature for a period of hours or perhaps days. Unfortunately, this technique was not

permissible with the dispersions examined here since the benzaldehyde is prone to oxidative attack. Prolonged standing would therefore have led to appreciable decomposition in some samples with a consequent reduction in the weight of benzaldehyde present. This, coupled with the appearance of by-products of the oxidation, would be likely to cause a shift in the position of the phase boundary. This effect has in fact been noted when old samples of benzaldehyde have been used. The addition of an antioxidant was not favoured since this would set up a four component system which would probably affect the phase equilibrium of the system.

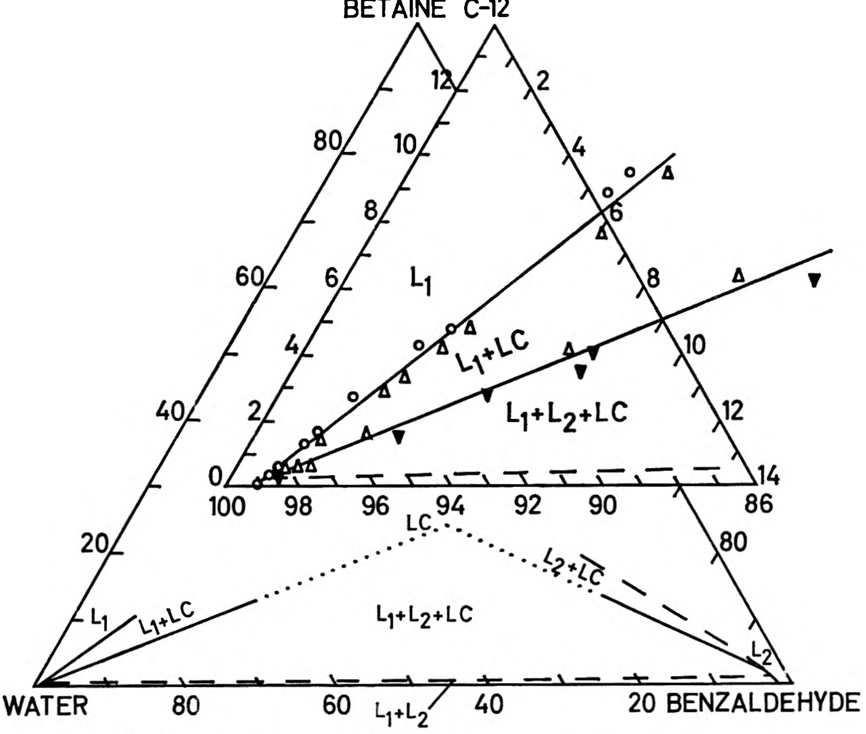

FIG. 4. System containing C_{12} betaine. Symbols as for Fig. 3.

The dispersions were therefore centrifuged and re-equilibrated as previously described in order to achieve separation as quickly as possible. Even so, in those dispersions containing appreciable amounts of the rapidly oxidising organic phase L_2, some loss of benzaldehyde must have taken place. This means that some phase boundaries could not be defined as accurately as others. For example, the extent of the L_1 and the $L_1 + LC$ phases were determined accurately and were reproducible, whereas the $L_1 + L_2$ boundaries were not so well defined.

Therefore, in the accompanying figures a continous line has been used to denote an accurately determined phase change. Those cases

determined with less certainty are shown by broken lines. The region containing L_1, L_2 and LC is triangular, the compositions of the three phases being given by the three corners of the triangle. If the slopes of the lines constituting this region are known, the corner points of the triangle can be calculated by extrapolation. All assumed phase boundaries of this type are shown by dotted lines.

In Figs. 1 to 6 the triangular insert is an enlargement of the lower left hand corner of the main diagram. The experimentally determined

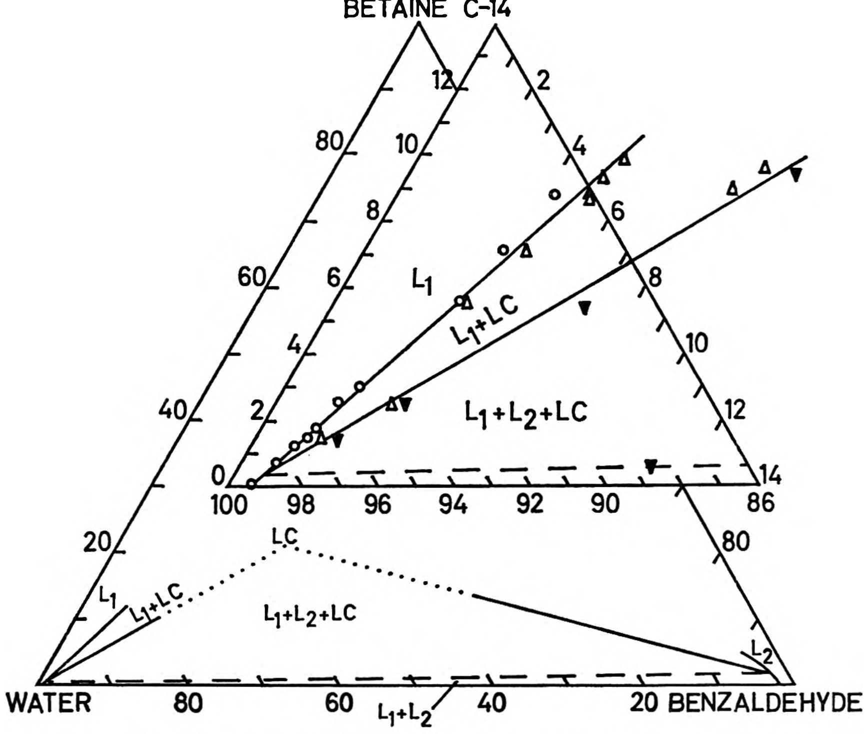

FIG. 5. System containing C_{14} betaine. Symbols as for Fig. 3.

points which have been used to define the phase boundaries are shown therein.

In the systems C_8 and C_{10} (Figs. 1 and 2) no liquid crystalline phase was observed. Thus all dispersions prepared in which the benzaldehyde was in excess of its solubility, when present in an aqueous solution of betaine, contained the two liquid phases, L_1 and L_2. It was found that the solubility of benzaldehyde increased when the alkyl chain, R, of the betaine was increased from 8 to 10 carbon atoms.

In the C_{11} system (Fig. 3) still more benzaldehyde was solubilised for the same weight percentage of betaine than in the above two systems. Further addition of benzaldehyde above the limit of the L_1 phase led to the

formation of a narrow L_1 + LC region, as distinct from the two liquid phases formed in systems C_8 and C_{10}. In addition a small triangular three phase region, $L_1 + L_2$ + LC, was found.

Some uncertainty exists as to the exact nature of the phases occupying the large area below the $L_1 + L_2$ + LC triangle. On occasions three liquids have been observed, although the third liquid may be a non-birefringent liquid crystalline phase. No attempt was made to investigate this region.

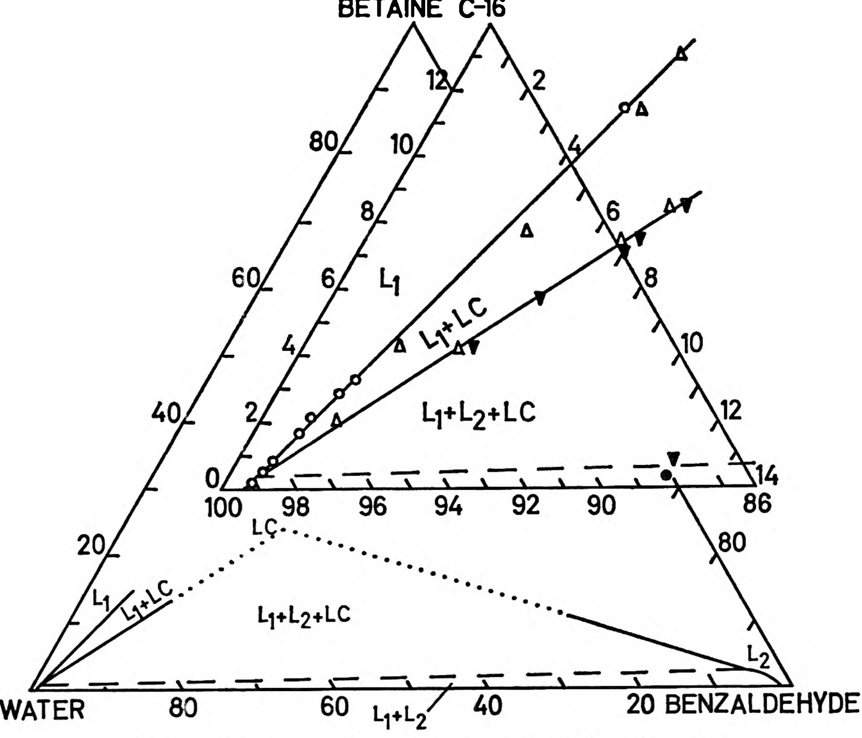

FIG. 6. System containing C_{16} betaine. Symbols as for Fig. 3.

Fig. 4 denotes the phase equilibrium boundaries which exist within the C_{12} system. When compared to system C_{11} (Fig. 3) it is seen that although the three phase region has been enlarged, the position of the L_1 + LC/$L_1 + L_2$ + LC boundary has undergone but little change. The L_1 + LC region has increased; however, at the same time, the extent of the L_1 phase has been reduced. Such a reduction is in contrast to the previous increases in the amount of benzaldehyde solubilised which occurred when the alkyl chain of the betaine molecule was changed from 8 to 10 and then to 11 carbon atoms.

In the systems C_{14} and C_{16} (Figs. 5 and 6) the aqueous solubility of benzaldehyde is progressively reduced as the higher betaine homologues

are used. Similarly, the appearance of the three phase region occurs at lower concentrations of benzaldehyde.

These results are best illustrated by reference to Table I, which shows the weight per cent of benzaldehyde necessary to cause phase changes in 5 and 10 per cent w/w aqueous solutions of the respective betaines at 25°. The weight per cent of benzaldehyde solubilised in the latter solutions are plotted against the number of carbon atoms in the betaine alkyl chain in Fig. 7.

TABLE I

WEIGHT PER CENT OF BENZALDEHYDE NECESSARY TO CAUSE PHASE CHANGES IN AQUEOUS BETAINE SOLUTIONS AT 25°

Ternary system	5 per cent w/w betaine		10 per cent w/w betaine	
	Weight per cent benzaldehyde		Weight per cent benzaldehyde	
C_8	1·9*	—	5·0*	—
C_{10}	5·7*	—	11·2*	—
C_{11}	7·8†	8·6‡	15·3†	16·3‡
C_{12}	3·8†	9·0‡	6·7†	17·0‡
C_{14}	3·0†	5·4‡	5·4†	10·3‡
C_{16}	2·5†	4·8‡	4·4†	9·1‡

* denotes a $L_1 \rightarrow L_1 + L_2$ phase change.
† denotes a $L_1 \rightarrow L_1 + LC$ phase change.
‡ denotes a $L_1 + LC \rightarrow L_1 + L_2 + LC$ phase change.

Table II shows the concentrations at which phase changes were observed in the four binary betaine-water systems C_{11}, C_{12}, C_{14} and C_{16} examined. In each case the phase transition was from isotropic $L_1 + LC$, the concentration at which this occurred decreasing as the betaine chain length was increased.

TABLE II

PHASE CHANGES IN BINARY BETAINE-WATER SYSTEMS AT 25°

Binary system	Concentration betaine per cent w/w	Phase change
C_{11}	47·7	$L_1 \rightarrow L_1 + LC$
C_{12}	43·1	$L_1 \rightarrow L_1 + LC$
C_{14}	34·4	$L_1 \rightarrow L_1 + LC$
C_{16}	24·2	$L_1 \rightarrow L_1 + LC$

DISCUSSION

The term amphiphile is used to describe substances possessing, within the same molecule, distinct regions of hydrophilic and lipophilic character (Winsor, 1954). That these molecules have opposing solubility tendencies is due to the presence of both polar and non-polar groups. Such a description covers a wide range of substances from the short chain alcohols, amines and carboxylic acids to the longer chain ionic surface-active agents or soaps. Whilst Winsor refers to all such compounds as amphiphiles Lawrence (1961) prefers to exclude the surface-active agents and reserve the term for those substances which are insoluble or poorly soluble in water alone.

It is necessary to distinguish between those amphiphiles which are either predominantly hydrophilic or lipophilic, and amphiphiles of intermediate molecular weight which show marked miscibility with both water and organic solvents. We define the former as O-amphiphiles and the latter as S-amphiphiles. O-amphiphiles are considered to be solutes which undergo "interaction solubilisation", a term suggested by Lawrence and Mills (1951), with the S-amphiphiles.

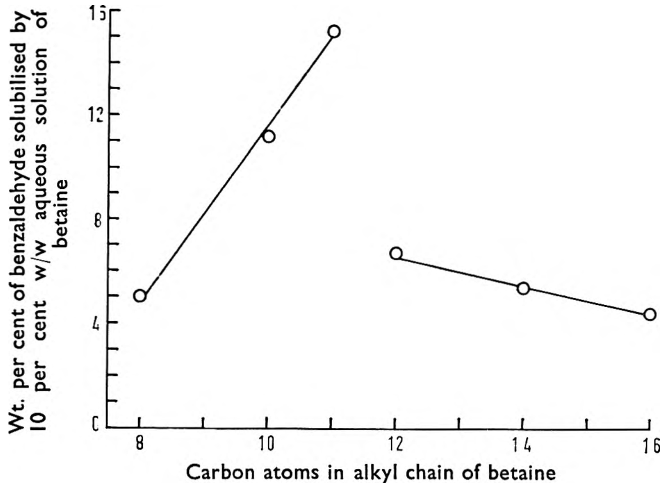

FIG. 7. Plot of weight per cent benzaldehyde solubilised in betaine solutions vs. the number of carbon atoms in the betaine alkyl chain.

Lawrence (1937) was the first to distinguish between the solubilisation of non-polar compounds and O-amphiphiles. With non-polar, water-insoluble materials such as hydrocarbons solubilisation takes place in the centre of the micelle. With O-amphiphiles, such as benzaldehyde, which is partially water-soluble, solubilisation occurs in the palisade layer of the micelle.

The continual addition of a non-polar liquid to an L_1 system containing micelles eventually causes the separation of another phase due to saturation of the interior of the micelles. The phase which separates contains excess non-polar liquid together with only negligible amounts of the other components of the system. When increasing amounts of an O-amphiphile are added the micelles themselves become sufficiently lipophilic, due to the inclusion of the additive in the palisade layer, to cause the separation of two phases (Klevens, 1950).

These effects are reflected in the behaviour of organic liquids when solubilised in aqueous solutions of S-amphiphiles of increasing molecular weight. In the case of non-polar organic liquids, Durand (1946) found increasing solubilisation of benzene by aqueous solutions of an homologous sodium n-alkanoate series, when the alkyl chain length was increased

over the range C_3 to C_{11}. Klevens (1950) has collected results for the solubilisation of ethylbenzene and n-heptane dispersed in solutions of potassium fatty acid soaps of increasing molecular weight which show a similar effect.

With O-amphiphiles a more complex variation in behaviour has been observed. Using different normal primary alcohols and amines, Harkins and Oppenheimer (1949) found, with most systems, an initial increase in the amount solubilised, which amount then tended to reach a constant value as the length of the fatty acid soap chain was increased further. With the C_{12} alcohol there was again an initial increase in solubility with increase in the length of the soap molecule, followed by a decrease with further increase in the number of carbon atoms in the fatty acid soap. These workers determined the solubility by a turbidimetric method, making the assumption that as the saturation point was exceeded the excess oil was emulsified; the solutions became therefore increasingly turbid. Unfortunately no attempt was made to identify the phases which separated. Both Hyde, Langbridge and Lawrence (1954) and Winsor (1954) have stressed the importance of identifying the nature of the phases separating in ternary systems since it is only when such phase changes are similar can valid comparisons as to solubility in different systems be made.

The complex variation in behaviour for liquid O-amphiphiles mentioned above is also shown by the betaine-benzaldehyde-water systems investigated in this paper. Fig. 7 shows that an essentially linear relationship exists between the weight of benzaldehyde solubilised and the alkyl chain length of the betaine up to, and including, the system C_{11}. Beyond this a decrease in solubility occurs but the relationship is still of a linear nature. This change in solubility coincides with the appearance of a relatively extensive $L_1 + LC$ region in the C_{12} system.

Lumb (1951) observed the formation of two isotropic liquid phases in equilibrium on the addition of octanol-1 to certain concentrations of potassium n-butyrate in water. Such a precipitation, according to Winsor (1954), resembles that in his Type I systems containing hydrocarbons. The apparent solubility of the alkanol in an alkanoate solution of given concentration would be expected to increase with the molecular weight of the alkanoate. The results obtained in this paper for benzaldehyde solubilised in systems C_8 and C_{10} are in agreement with this prediction.

In the systems C_{12}, C_{14} and C_{16}, the addition of benzaldehyde to aqueous betaine solutions leads to the precipitation of the two phases $L_1 + LC$, and a reduction in the solubility of the benzaldehyde. Winsor (1954) has noted that with certain polar organic liquids, where solubilisation is limited by the formation of such an $L_1 + LC$ system, an increase in the molecular weight of the surface-active agent produces a reduction in the apparent solubility of the solubilisate. The decrease in solubility found with the systems studied here is paralleled by the decreasing concentration at which the L_1 to $L_1 + LC$ phase transition takes place in the four binary systems C_{11}, C_{12}, C_{14} and C_{16}. It is presumably this transition

in the binary system which reduces the solubility of benzaldehyde in the ternary system by progressively reducing the area of the L_1 phase.

In the system C_{11} the increase in the amount of benzaldehyde solubilised per CH_2 group has been maintained. However the nature of the phase change is such that, in the light of the previous discussion, a reduction in the apparent solubility of benzaldehyde would have been expected. The formation of the narrow $L_1 + LC$ region in this system has had, therefore, little or no effect on the amount solubilised.

As mentioned earlier a dispersion consisting of three liquid phases has been observed in this system, in an area where, with the higher betaine homologues, the ternary $L_1 + L_2 + LC$ triangle exists. A similar situation has been observed by Mulley and Metcalf (1961). It may be that the third liquid is in fact a non-birefringent liquid crystalline phase.

These facts suggest that system C_{11} is of a transitional nature and offer some explanation for the results obtained.

The relevant parts of the ternary phase diagrams discussed above were determined in an attempt to rationalise the study of the oxidation of benzaldehyde when dispersed in solutions of betaines. In previous oxidation studies it had been assumed that all material in excess of its solubility in aqueous solutions of surface-active agents formed dispersions containing two liquid phases. In the systems studied in this investigation such a situation was found to exist only when the C_8 and C_{10} betaine homologues were used. The presence of a liquid crystalline phase in the other systems studied obviously invalidates an assumption of this nature.

Investigations which are concerned with the effect of the different phases found in these systems on the oxidation rate of benzaldehyde will form the subject of future communications.

Acknowledgement. The authors wish to thank Dr. B. A. Mulley for many helpful discussions.

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THE EFFECTS OF GUANETHIDINE ON THE NORADRENALINE CONTENT OF THE HYPOTHALAMUS IN THE CAT AND RAT

BY R. DAGIRMANJIAN*

From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

Received February 28, 1963

Single injections of guanethidine 15 mg./kg. i.p. did not lower the hypothalamic noradrenaline in cats, but, daily injections of 15 mg./kg. s.c. over a 7 day period consistently produced a decrease in the hypothalamic noradrenaline. Attempts to lower the hypothalamic noradrenaline in rats after single injections or daily injections of guanethidine were unsuccessful.

GUANETHIDINE, 2-(octahydro-1-azocinyl) ethyl guanidine sulphate, is a hypotensive drug, which produces a depletion in the noradrenaline content in the peripheral tissues (Butterfield and Richardson, 1961; Cass, Kuntzman and Brodie, 1961; Cass and Spriggs, 1961; Sanan and Vogt, 1962). Although the levels of noradrenaline are not reduced to the extent necessary to block impulse transmission, this depletion possibly contributes to its hypotensive action.

Conflicting reports occur about whether guanethidine causes a depletion of noradrenaline in the brain. Cass and Spriggs (1961) were unable to demonstrate a change in the noradrenaline content of the cat or rabbit brain and attributed this lack of an effect to the inability of the highly ionised molecule to penetrate the blood-brain barrier. Significant decreases have been reported recently in the rat brain within 1–3 hr. after acute administration of guanethidine (Pfeifer, Vizi and Satory, 1962). Sanan and Vogt (1962) in some but not all experiments observed decreases in the hypothalamic noradrenaline of the cat and rabbit. This effect was inconsistent from animal to animal and especially from colony to colony. This suggested a possible reflex stimulation of the sympathetic centres and not a direct effect.

Further experiments are reported here on the effect of acute and chronic administration of guanethidine in cats and rats on the content of noradrenaline in the hypothalamus.

METHODS

Male or female cats, bred in Babraham and weighing 1·7–2·8 kg. were bled under chloroform anaesthesia. The tissues were removed quickly, weighed, and placed into acid-ethanol (0·1 ml. conc. HCl to 100 ml. ethanol) chilled in a dry ice-acetone mixture until extracted. Female albino rats, 175–225 g., were decapitated; the hypothalamus removed, weighed and placed into chilled acid ethanol. The portion of hypothalamus removed excluded the corpora mammillaria and optic tracts.

The tissues were extracted and purified using procedures previously described (Vogt, 1952, 1953, 1954). Noradrenaline was determined by

* Present address: Department of Pharmacology, The University of Rochester, Rochester, New York, U.S.A.

the bioassay method using the pithed rat blood pressure (Muscholl and Vogt, 1957.) Recoveries were checked in some experiments by adding 0·1 μg. noradrenaline to about 0·1 g. of cerebellar tissue in which the concentration of noradrenaline is known to be less than 0·1 μg./g. (Vogt, 1954). There was no pooling of samples, the tissue of each animal being extracted singly.

Guanethidine sulphate was injected as a 1–2 per cent solution in 0·9 per cent saline. Doses are expressed in terms of the weight of the salt. In acute experiments in both cats and rats, injections were given intraperitoneally. For chronic administration the solution was "sterilised" by bringing it to the boil. In chronic experiments in cats the "sterilised" solution was given subcutaneously, and in rats it was injected subcutaneously.

Results

At various times up to 72 hr. after a single injection of guanethidine, 15 mg./kg., in cats, the hypothalamic content of noradrenaline was not lowered but in the superior cervical ganglia the content of noradrenaline was significantly lowered (Table I). Yet examination of the ranges of

TABLE I

NORADRENALINE CONTENT IN HYPOTHALAMUS AND SUPERIOR CERVICAL GANGLIA OF CATS AT VARIOUS TIMES AFTER GUANETHIDINE, 15 MG./KG., I.P. NORADRENALINE IN μG./G. FRESH TISSUE, MEAN ± S.E. OF THE MEAN CORRECTED FROM RECOVERIES OF 50–70 PER CENT. RANGE IN BRACKETS. *SIGNIFICANT DECREASES ($P<0.05$) TESTED BY "t" TEST ANALYSIS

Time interval (hr.)	No. of animals	Hypothalamus	Superior cervical ganglia
0	5	2·3 ± 0·19 (1·8–2·9)	10·6 ± 1·5 (6·9–12·2)
4	2	1·8 ± 0·24 (1·6–2·1)	5·2 ± 0·7* (5·1–5·3)
16	5	1·6 ± 0·30 (0·71–2·7)	4·0 ± 1·2* (1·2–7·0)
24	2	2·6 ± 0·94 (1·7–3·6)	4·0 ± 1·2* (2·9–5·1)
72	3	2·4 ± 0·14 (2·2–2·8)	7·2 ± 1·4* (4·3–9·2)
7 day chronic 15 mg./kg. s.c.	5	0·85 ± 0·12* (0·60–1·1)	4·0 ± 0·54* (2·0–4·9)

the results for the hypothalamus indicated individual variations and single figures which were low. This was especially true 16 hr. after guanethidine when the range was 0·71–2·7 μg./g. To rule out the possibility that larger doses may have depleted more effectively, a cat was given 25 mg./kg., but 16 hr. later the noradrenaline content of the hypothalamus of this animal was 2·5 μg./g., near the upper limit of the normal range. In contrast, daily injections over a 7 day period consistently produced a significant decrease in the hypothalamic noradrenaline (Table I).

Overt signs in these cats indicated depression of the sympathetic nervous system. After a single injection there was a partial relaxation of the nictitating membrane, slight miosis, some closure of the palpebral fissure, and some reduction in motor activity. No phase of early excitement was evident in these cats. All cats had diarrhoea. With the chronic treatment

similar signs of partially relaxed membrane, slight miosis, and a generally quiet demeanour were present. In addition, there was some anorexia but little loss of weight. After 3 days, two of the cats showed marked restlessness and resistance to petting; on the last day, they appeared apprehensive and, if excited by the presence of strangers, signs of adrenal medullary stimulation (mydriasis, contraction of the nictitating membrane, and panting) became evident.

In one experiment with rats, a dose of 5 mg./kg. was given and the hypothalamus removed after a time interval of 3 hr. This procedure was the same as that of Pfeifer, Vizi and Satory (1962). In an additional group of rats an injection of 15 mg./kg. was given. There was no effect on the hypothalamic noradrenaline after a single dose of guanethidine and in other experiments there was no effect after chronic administration (Table II). Yet in the group receiving 5 mg./kg. once, there was a wider range (0·56–1·43 μg./g.) of noradrenaline contents than in control rats, as noted in cats. The only signs observed in rats was diarrhoea.

TABLE II

NORADRENALINE CONTENT OF THE HYPOTHALAMUS IN RATS AFTER SINGLE AND DAILY SUBCUTANEOUS INJECTIONS OF GUANETHIDINE. CONTROLS INJECTED WITH 0·9 PER CENT NACL SOLUTION. NORADRENALINE IN μG./G. WET TISSUE, MEAN ± S.E. OF THE MEAN. RANGE IN BRACKETS

Dose of guanethidine (mg./kg.)	3 hr. after single injection		24 hr. after the last of 7 daily injections	
	μg./g.	No. of rats	μg./g.	No. of rats
0	1·2 ± 0·11 (0·91–1·46)	5	1·4	1
5	1·1 ± 0·12 (0·56–1·40)	6		
15	1·2 ± 0·08 (1·0–1·40)	4	1·4 ± 0·04 (1·3–1·5)	5

The failure to obtain consistent effects on the noradrenaline content of the hypothalamus of the rat with sufficient guanethidine to cause a clear depletion in the cat, prompted experiments with another drug, β-tetrahydronaphthylamine, which, by stimulating sympathetic centres, regularly lowers the hypothalamic noradrenaline in the cat (Vogt, 1954).

Four rats were given β-tetrahydronaphthylamine, 30 mg./kg., subcutaneously and killed 4 hr. later. The signs of sympathetic stimulation seen were exophthalmos, piloerection, restlessness, rise in temperature, and increased rate of respiration. The hypothalamic content of noradrenaline was decreased to 0·88 ± 0·08 μg./g., but this difference was not statistically significant from the central means of 1·2 ± 0·11. The result suggests that stimulation of the sympathetic centres by β-tetrahydronaphthylamine does not affect the brain noradrenaline of the rat as readily as that of the cat.

DISCUSSION

Sanan and Vogt (1962) suggested a reflex stimulation of the sympathetic centres to explain the occasional decrease of the hypothalamic noradrenaline after a single dose of guanethidine. The consistent fall in the

hypothalamic content of noradrenaline after giving repeated daily doses of guanethidine is compatible with this idea. The effect was seen in the cat which reacts to stimulation of the sympathetic centres by β-tetrahydronaphthylamine with a fall in brain noradrenaline (Vogt, 1954). This effect of guanethidine was absent in the rat in which the hypothalamic noradrenaline was also more resistant to depletion by β-tetrahydronaphthylamine.

Another possibility is that after prolonged administration, sufficient quantities of guanethidine may pass the blood-brain barrier to exert a direct effect in depleting noradrenaline from the hypothalamus. Kaneko, McCubbin and Page (1962) conducted experiments in dogs to test the central effect of guanethidine. In their experiments, systemic injections of guanethidine were ineffective, but injections given intraventricularly or intracisternally caused a central inhibition of vasomotor tone. These workers suggested that prolonged clinical use of guanethidine could result in a situation where enough of the drug passed the blood-brain barrier to exert an effect on the cardiovascular system.

The third possibility is that the action of guanethidine is, indeed, indirect, but due to enhanced central sympathetic activity elicited as a result of lack of a feed-back mechanism. The centres might be overactive in response to the inhibition by guanethidine of the peripheral adrenergic neurones.

The report by Pfeifer, and others (1962) that brain noradrenaline was depleted after one dose of guanethidine in the rat was not confirmed. The difference in the results can hardly be attributed to the fact that I was measuring the noradrenaline in the hypothalamus, rather than in the whole brain. Other drugs which have been tested produce similar losses of noradrenaline in the midbrain and in the hypothalamus (Vogt, 1954), and these two regions together contain a large fraction of the noradrenaline of the whole brain. There is, however, the possibility that different strains of rats react differently. In addition, different methods of extraction were used.

Acknowledgements. The research reported in this paper has been sponsored in part by the Air Force Office of Scientific Research, OAR, through the European Office, Aerospace Research, United Air Force. This work was performed during the tenure of a Riker Postdoctoral Fellowship under the guidance and direction of Dr. M. Vogt. Ciba Laboratories Ltd. kindly supplied the guanethidine used in these experiments.

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SOME PHYSICO-CHEMICAL STUDIES OF LYSOPHOSPHATIDYLETHANOLAMINE SOLS

BY D. C. ROBINS AND I. L. THOMAS

From the Welsh School of Pharmacy, Welsh College of Advanced Technology, Cathays Park, Cardiff

Received February 25, 1963

The solubilities of lysophosphatidylethanolamine in some organic solvents have been determined. Surface tension measurements of aqueous sols showed it to have pronounced surface-active properties. There was a surface ageing effect which was followed by a rise in the surface tension. Possible explanations for these effects are given. The effects of concentration, pH, mono- and divalent salts on the surface activity have been investigated. The critical micelle concentration was in the range 0·001 to 0·002 per cent w/v. The isoelectric point was at pH 3·25. The stability of sols of the phosphatide in the presence of mono- and divalent salts has also been examined.

LYSOPHOSPHATIDYLETHANOLAMINE (lysoPE) is obtained from phosphatidylethanolamine (PE) by the hydrolysis of one fatty acid ester linkage which can be effected by the enzyme phospholipase A, an enzyme found in the venom of vipers. The specific site of action has been established as the β ester position of PE (Tattrie, 1959; Hanahan, Brockerhoff and Barron, 1960, de Haas and van Deenen, 1961a). The zwitterionic structure of Lα-lysoPE is shown by (I)

$$\begin{array}{l} R{\cdot}COO{\cdot}CH_2 \\ \quad\quad\quad | \\ \quad\quad H{\cdot}C{\cdot}OH \\ \quad\quad\quad | \\ \quad\quad CH_2{\cdot}O{\cdot}\overset{O}{\overset{\|}{P}}{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{N}H_3 \\ \quad\quad\quad\quad\quad\; \underset{O^-}{|} \end{array}$$

I

—where R is a predominantly saturated hydrocarbon chain containing mainly 15 or 17 carbon atoms.

The molecule is amphipathic since it contains a hydrophobic non-polar hydrocarbon chain, and a hydrophilic, polar, phosphate-ethanolamine grouping. Therefore, it would be expected to show surface-active properties.

EXPERIMENTAL

Preparation of LysoPE

The method of preparation was based on that of Long and Penny (1957).

PE was first prepared by the method of Robins and Thomas (1963). PE (1·65 g.) was dissolved in ether (165 ml.) and Russell Viper venom (10 mg. in 4 ml. water) added. The pH of the solution was adjusted to 7·0 with ammonium hydroxide. The precipitation of lysoPE from the solution commenced after about 24 hr. and was complete after 3 days.

The ether was decanted from the pale buff precipitate, and the water removed by shaking with successive small volumes of acetone.

The sample was purified by dissolving in the minimum quantity of methanol at 55°, centrifuging to remove the venom, and precipitating by adding ether, in which residual PE is soluble. This was repeated 4 times to yield 0·6 g. of a pure white, microcrystalline, non-hygroscopic powder, having nitrogen: phosphorous ratio 1:0·96, and an iodine value of 2. The product was dissolved in methanol, and stored under nitrogen at −20°.

Solubility Studies

The solubility of lysoPE in some organic solvents was determined over the temperature range 25–50°. The method was that of Robins and Thomas (1963). The results are in Table I.

TABLE I

SOLUBILITY OF LYSOPE IN SOME ORGANIC SOLVENTS

Solvent	Solubility in g./100 ml. solution at:					
	25°	30°	35°	40°	45°	50°
Methanol	0·365	0·415	0·445	0·510	0·530	0·565
Ethanol	0·060	0·180	0·250	0·280	0·365	0·415
Chloroform	0·085	0·095	0·110	0·135	0·140	0·155
Methyl ethyl ketone	0·010	0·010	0·010	0·020	0·020	0·030
Acetone	0	0·010	0·010	0·015	0·015	0·020
Ether	0·005	—	—	—	—	—

Surface Tension Studies

Preparation of aqueous sols. A weighed amount of lysoPE was dissolved in a small volume of purified water by vigorous shaking. The sol was passed through an ion-exchange column (Robins and Thomas, 1963) and made up to volume.

Slight variations occurred in the values of the surface tension with sols of the same concentration, owing to the inherent errors in the method of preparation. To overcome these, sufficient sol was prepared for a series of experiments and portions used as required. Since the surface tension varied with the age of a lysoPE sol, the bulk sol was divided into portions and frozen at −20°. Portions were melted and used as required; freezing and melting did not affect the surface tension.

Apparatus. A static method (Wilhelmy plate) was used for surface tension measurements, as described by Robins and Thomas (1963).

Variation of surface tension with time. A 0·005 per cent w/v lysoPE sol was prepared, and the variation of its surface tension with time was investigated. There was initially a rapid fall in the surface tension, the rate of fall progressively decreasing, until eventually a steady value was reached in 3 to 6 hr. (Fig. 1). On subsequent days, the minimum surface tension value of the same sol was higher on each successive day.

The variation of surface tension with time for sols of different concentrations is given in Fig. 2. With the most dilute sols, the surface tension was still falling slightly after 5 hr., but with increase in concentration

the time required to reach a minimum value decreased. The values of the surface tension after 6 hr. for these sols are given in Fig. 3.

Variation of surface tension of lysoPE sols with pH. 0·005 per cent w/v lysoPE sols were prepared, various amounts of hydrochloric acid added, and the pH measured with a Dynacap pH meter. The equilibrium values of the surface tension of these sols, and the times required to reach them are given in Table II.

TABLE II

VARIATION OF THE SURFACE TENSION OF 0·005 PER CENT W/V LYSOPE SOLS WITH pH

pH	Time to reach equilibrium (hr.)	Equilibrium value of surface tension (dynes/cm.)
6·1	5·25	34·27
5·42	2·75	32·75
4·83	2	31·71
4·30	1·5	30·11
3·82	0·75	29·70
3·1	0·5	28·80
2·3	1	30·40
2·02	1·75	31·51
1·7	3	32·00

The surface tension of the sol at pH 2·3 was measured on successive days and, within the limits of experimental error, the values were found to be identical on each day (Table III).

TABLE III

VARIATION OF THE SURFACE TENSION OF A 0·005 PER CENT W/V LYSOPE SOL WITH TIME AT pH 2·3

Time (hr.)	Surface tension (dynes/cm.)		
	1st day	2nd day	3rd day
0·5	30·41	30·21	29·78
1	30·10	29·27	29·01
2	29·44	28·80	28·53
3	29·23	28·76	28·40
4	29·00	28·74	28·46
5	29·04	28·70	28·40
6	28·98	28·70	28·40

Sol agitated at the beginning of each day.

With a 0·005 per cent w/v sol, the pH of which had been adjusted to 10·5 with sodium hydroxide, the surface tension fell on the first day, reaching a minimum in 1 hr.; it then rose, reaching a constant value after 4½ hr. On subsequent days, no minimum was obtained, but there was a prolonged surface-ageing effect; the surface tension after 6 hr. being lower on each successive day (Fig. 4).

Variation of surface tension of lysolecithin sols with pH. The effect of time on the surface tension of a 0·0075 per cent w/v lysolecithin sol at pH values of 6·5, 1·6 and 10·1 was also examined.

At pH 6·5 there was a small surface-ageing effect, not followed by a rise in the surface tension, which confirmed Robinson and Saunders' (1958) findings. At pH 1·6, the surface tension fell about 0·75 dyne/cm.

over 2 hr., after which it remained constant. At pH 10·1, the surface tension rose 1–2 dynes/cm. to a maximum after 3 hr., and then fell slightly. On the following day, on the same sol, no maximum was exhibited, and the surface tension was lower and fell continuously (Table IV).

TABLE IV

VARIATION OF THE SURFACE TENSION WITH TIME OF A 0·0075 PER CENT W/V LYSOLECITHIN SOL AT pH 10·1

Time (hr.)	Surface tension (dynes/cm.)	
	1st day	2nd day
0·25	38·50	34·70
0·5	39·27	34·07
1	39·82	33·40
2	40·21	32·65
3	40·29	32·46
4	40·04	32·08
5	39·93	32·02

Effect of salts on surface tension. The effects of potassium and calcium chlorides on the surface tension of 0·005 per cent w/v lysoPE sols were investigated. The minimum values and the times taken to reach them are given in Table V.

TABLE V

EFFECT OF SALTS ON THE SURFACE TENSION OF 0·005 PER CENT W/V LYSOPE SOLS

Molar conc. of KCl	Minimum surface tension values (dynes/cm.)	Time taken to reach the minimum value (hr.)
—	36·07	3·25
1×10^{-5}	33·13	2·5
1×10^{-4}	32·94	2·75
1×10^{-3}	33·23	2·75
1×10^{-1}	33·69	2·75
Molar conc. of $CaCl_2$		
1×10^{-6}	35·96	0·5
1×10^{-5}	35·80	0·75
1×10^{-4}	37·28	1
1×10^{-3}	38·23	1·75
1×10^{-2}	39·63	1·75

Effect of salts on the stability of lysoPE sols. One ml. of a 0·05 per cent w/v lysoPE sol was placed in each of a series of sample tubes. Potassium chloride 5×10^{-1} to 1×10^{-5}M or calcium chloride 1×10^{-2} to 1×10^{-6}M solutions were added by Agla micrometer syringe, and the volume adjusted with water to a final concentration of 0·04 per cent w/v lysoPE in each tube.

A heavy precipitate occurred with 5×10^{-1} and 2×10^{-1}M potassium chloride and with 1×10^{-2}M calcium chloride and a weak precipitate with 5×10^{-3}M calcium chloride. There was no precipitation at the other concentrations.

DISCUSSION

LysoPE has been prepared by the direct action of venom, as reported by Long and Penny (1957) and de Haas and van Deenen (1961a, 1961b). No difficulty was encountered in obtaining lysoPE in this way, provided

that 72 hr. were allowed for complete reaction at room temperature, despite references to the impossibility of the method (Chargaff and Cohen, 1939; Lea, Rhodes and Stoll, 1955). A slow degradation of the phosphatide prefaced by a lag period of 24 hr. noticed by Davidson, Long and Penny (1955) and the need to adjust the reaction mixture to pH 7 as found by Long and Penny (1957) may be the reasons for the differing reports.

The solubility of lysoPE in organic solvents showed a similar pattern to the figures quoted for lysolecithin (Robinson and Saunders, 1958), but the values were lower. This was probably because of the larger net charge on the lysoPE molecule. The results are in accord with previous indications (Levene, Rolf and Simms, 1924). The solubility of lysoPE in water was low. A 0·05 per cent w/v sol was turbid, and was obtained only after prolonged shaking at 40°.

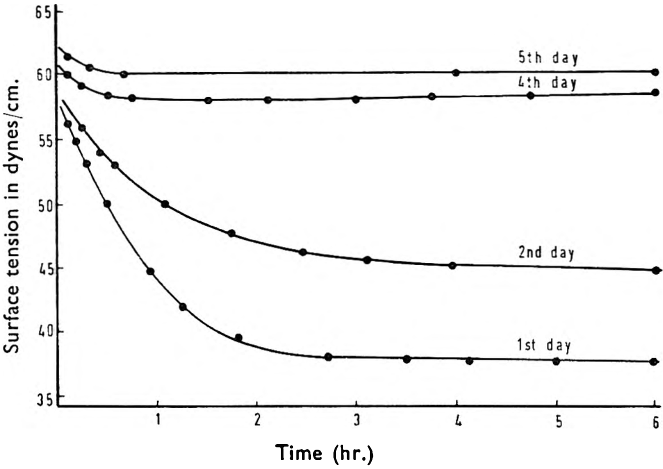

FIG. 1. Variation of surface tension of a 0·005 per cent w/v lysoPE sol with time.

LysoPE sols showed a surface-ageing effect, but this was not so pronounced as that shown by PE sols. As the concentration of the sol was increased, the surface-ageing effect decreased, and was very small above the critical micelle concentration. The possible explanations for the surface ageing in PE sols have already been discussed (Robins and Thomas, 1963). In lysoPE sols we consider the main factor is the existence of an electrical double layer at the surface. Evidence supporting this view is, firstly, that the ageing effect in lysoPE, where the surface-active ion has a distinct net negative charge, is much greater than in lysolecithin which only has a very small net negative charge. Secondly, the surface tension studies on lysoPE sols at various acidic pH values have shown (Table II) that as the net charge on the molecule is reduced, the surface-ageing effect is reduced, reaching a minimum at the isoelectric point.

The second possible factor causing the ageing effect is the time required for the molecules to orientate themselves at the surface. The lysoPE

molecules are less bulky than the PE molecules, and the fatty acids are more saturated, thus enabling them to orientate more readily at the surface. This would account for the shorter surface-ageing effect.

In Fig. 3, the surface tensions of sols after 6 hr. are plotted against the various concentrations. There is a sharp change in the slope of the graph between 0·001 and 0·002 per cent w/v indicating that the critical micelle concentration for lysoPE occurs within this concentration range.

Upon plotting the values of surface tension against pH, a minimum in the curve occurred at pH 3·25, indicating this pH value to be the isoelectric point for lysoPE. At the isoelectric point, the net charge on the molecule would be at a minimum, and consequently the molecules would pack more closely in the surface, causing the greatest lowering of the surface tension. As would have been expected, the isoelectric point of lysoPE is very similar to that of PE.

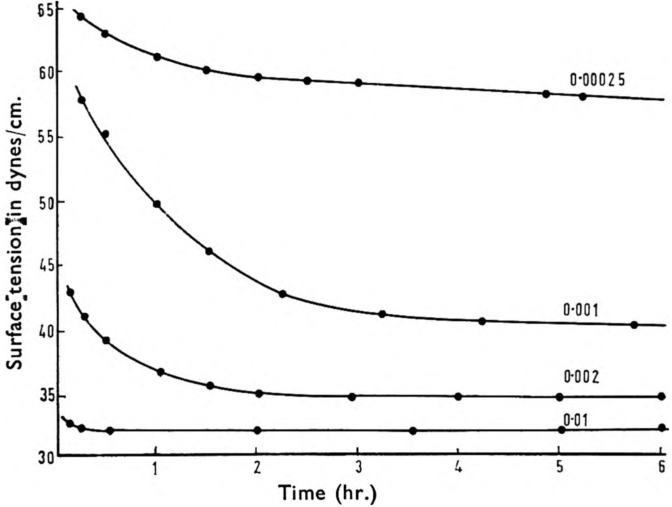

FIG. 2. Variation of surface tension with time of lysoPE sols of varying concentration. Figures on the curves are concentrations in per cent w/v.

There are a number of possible explanations for the changes in surface tension of lysoPE sols with time at various pH values. Firstly, they may be due to hydrolysis of one or more of the ester linkages present in the molecule. Secondly, they may result from an intramolecular rearrangement, whereby the fatty acid chain migrates from the α- to the β-position. Thirdly, they may be due to migration of the phosphate-ethanolamine moiety from the γ- to the β-position.

Whilst hydrolysis of the ester linkages probably occurs in alkaline conditions it is doubtful whether this is the cause of the change in surface tension at neutral pH, since were it so, PE, lysoPE and lysolecithin would behave similarly, and no such change occurs with PE or lysolecithin sols.

An intramolecular migration is likely, since there is a free hydroxyl group on the β-carbon atom. In PE, this hydroxyl group is esterified

with another fatty acid, thus rendering migration impossible. It has been found that after an initial surface-ageing effect, the surface tension of PE sols remained constant (Robins and Thomas, 1963).

A possible mechanism for the fatty acid migration would be as follows:

$$
\begin{array}{lcl}
CH_2{-}O{-}C(=O^{\delta-})\cdot R & & CH_2{-}O{-}C(OH)\cdot R \\
| & \rightleftharpoons & |\quad / \\
CH{\cdot}OH^{\delta+} & & CH{\cdot}O \\
| & & | \\
CH_2{\cdot}O{\cdot}P(=O)(O^-){\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{N}H_3 & & CH_2{\cdot}O{\cdot}P(=O)(O^-){\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{N}H_3
\end{array}
\rightleftharpoons
\begin{array}{l}
CH_2{\cdot}OH \\
| \\
CH{\cdot}O{\cdot}CO\cdot R \\
| \\
CH_2{\cdot}O{\cdot}P(=O)(O^-){\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{N}H_3
\end{array}
$$

Hanahan and Uziel (1957) have reported that a migration of the fatty acid in lysolecithin occurred in the presence of either a migratase enzyme or 0·05N hydrochloric acid. However, we have found in acid solution with both lysolecithin and lysoPE that once an equilibrium value is reached after the initial surface-ageing effect, the surface tension does not change. Assuming the above mechanism to be correct, in acid conditions the slight ionisation of the β-alcoholic grouping would be suppressed and thus prevent migration. In alkaline conditions, the ionisation would be increased and would thus favour migration, with which our results agree. However, the argument against fatty acid migration is the failure of lysolecithin to show a similar rise to lysoPE in surface tension with time at neutral pH values.

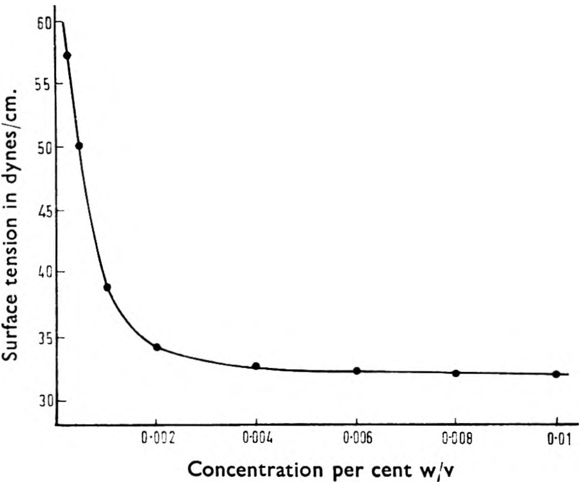

FIG. 3. Variation of surface tension of lysoPE sols with concentration.

The third possibility is the migration of the phosphate-ethanolamine grouping from the γ- to the β-position. Many workers have studied the effects of acid and alkali on glycerophosphoric acid (GPA). Bailly (1938, 1939) has shown that when β-GPA is treated with acid, some is

converted into α-GPA without any liberation of phosphoric acid or glycerol, and, in the equilibrium mixture obtained, the α-form predominated. Verkade, Stoppelenburg and Cohen (1940) confirmed Bailly's findings and Chargaff (1942) showed that the conversion of β-GPA to α-GPA was an intramolecular rearrangement by treating β-GPA with acid in the presence of radioactive sodium phosphate. The isolated α-GPA contained no radioactive phosphorous. Bailly and Gaumé (1934a, 1934b) have reported that α-GPA is partially converted into β-GPA in alkaline conditions, and that the β-form predominates in the equilibrium mixture. Baer and Kates (1948) have shown that on acid or alkaline hydrolysis of glycerylphosphorylcholine, a reversible α to β migration of the phosphoric acid grouping occurs, accompanied by the liberation of choline. They also found that in N hydrochloric acid the α-form predominated (91 per cent), whilst in N sodium hydroxide the β-form predominated (56 per cent). We are not aware of any report of a migration of the phosphate-base grouping in an intact phosphatide molecule. A possible mechanism for such a migration in lysoPE would be as follows:

$$\begin{array}{l} CH_2\cdot O\cdot COR \\ | \\ CH\cdot OH \\ | \\ CH_2\cdot O\cdot \overset{O}{\overset{\|}{P}}\cdot O\cdot CH_2\cdot CH_2\cdot \overset{+}{N}H_3 \\ \qquad\quad\; | \\ \qquad\quad\; O^- \end{array} \rightleftharpoons \begin{array}{l} CH_2\cdot O\cdot COR \\ | \\ CH{-}O \quad OH \\ | \qquad\;\backslash\,/ \\ CH_2\cdot O\cdot P\cdot O\cdot CH_2\cdot CH_2\cdot \overset{+}{N}H_3 \\ \qquad\quad\; | \\ \qquad\quad\; O^- \end{array} \rightleftharpoons \begin{array}{l} CH_2\cdot O\cdot COR \\ | \\ CH\cdot O\cdot \overset{O}{\overset{\|}{P}}\cdot O\cdot CH_2\cdot CH_2\cdot \overset{+}{N}H_3 \\ | \qquad\;\; | \\ CH_2\cdot OH \;\; O^- \end{array}$$

Here again, in acid solution the slight ionisation of the alcoholic grouping would be suppressed, thus reducing the possibility of migration. However, it has been reported that a little of the phosphoric acid in α-GPA does migrate to the β-position even in acid solution. Thus a small migration of the phosphate-base grouping may be occurring in lysoPE and lysolecithin sols, but the consequent change in surface tension may be too small to be detected. Increased ionisation of the alcoholic grouping in alkaline solution would favour migration, which the results with lysoPE and lysolecithin sols agree.

The failure of an intramolecular migration to occur in lysolecithin in a neutral aqueous sol, may be explained by a ring structure being formed by the equivalently highly ionised phosphoric acid and choline groupings, thus:

$$\begin{array}{l} CH_2\cdot O\cdot CO\cdot R \\ | \\ CH\cdot OH \\ | \qquad\quad O \;\; O{-}CH_2 \\ CH_2O{-}P \qquad\quad CH_2 \\ \qquad\quad O^-\cdots \overset{+}{N}Me_3 \end{array}$$

A model of such a molecule shows that it is possible for such a ring structure to sterically prevent the migration of the phosphate-choline moiety.

In a neutral aqueous lysoPE sol such a ring structure would be less

likely to be formed, since the amino-group of ethanolamine would be only slightly ionised, and hence migration could occur.

The complex time effect exhibited by the lysoPE sols at pH 10·2 can be explained as follows. Initially the surface-ageing effect more than offsets the rise in surface tension due to intramolecular migrations so that the surface tension falls. After 1 hr. it rises because the migration has now become the predominant factor. After 4·5 hr. an equilibrium between the α- and β-forms is reached and the surface tension remains constant. The unbroken fall found with the same sol on subsequent days is probably due to the slow hydrolysis of the ester linkages. The fatty acids released would form soaps with the sodium hydroxide present, and thus cause the surface tension to fall slowly.

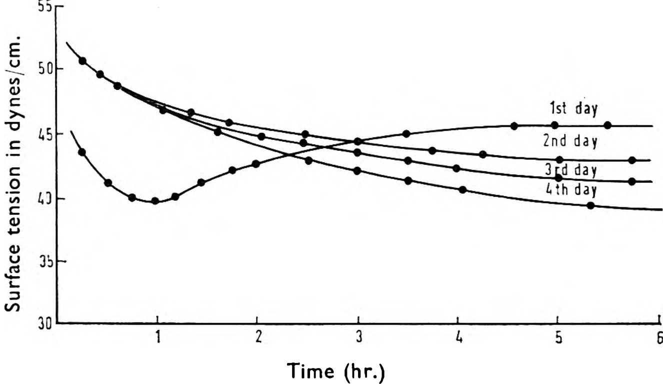

FIG. 4. Variation of surface tension of a 0·005 per cent w/v lysoPE sol with time at pH 10·5.

The time effect shown by a 0·0075 per cent w/v lysolecithin sol at pH 10·1 may be explained similarly, except that no initial fall in surface tension occurred, since the small surface-ageing effect is masked by the rise in surface tension due to the intramolecular migration.

Neither calcium nor potassium ions prevented the change in surface tension values of lysoPE sols with time. Hence the expression "minimum" values are used rather than "equilibrium" values to indicate the lowest values obtained from the first day's readings.

It has been reported (Nutting, Long and Harkins, 1940) that the presence of electrolytes reduces the surface-ageing effect, and the higher the concentration and valency of the ions, the greater is the reduction. With lysoPE sols the ageing effect was found to be dependent on the minimum value of the surface tension rather than the electrolyte concentration. The lower the minimum value the shorter the ageing effect. The results in Table V also show that the higher the valency of the added cation, the shorter was the surface-ageing time.

The presence of various concentrations of potassium chloride caused a slight fall in the surface tension of lysoPE sols. These results are as expected, since potassium ions, being univalent, are not capable of linking

two lysoPE molecules together, and thus do not improve their packing at the surface. An increase in the concentration of calcium chloride caused a slight rise in the surface tension of the sols. This is the reverse of what would have been expected, since any linkage between the calcium ion and the phospholipid molecules would have facilitated closer packing in the surface. However, Robinson and Saunders (1958) found that calcium chloride caused a similar rise in the surface tension of lysolecithin sols.

Concentrations of 5×10^{-3}M and above of calcium chloride caused flocculation of the lysoPE sols, whilst concentrations of 2×10^{-1}M and above of potassium chloride were needed. Since the surface tension results did not indicate conclusively that any interaction took place between the salts and lysoPE, it is probable that the flocculation was due to a salting-out effect. Thus, it would be expected that the divalent calcium ions would be more effective than the monovalent potassium ions. It is interesting to compare these results with those obtained by Saunders (1957), who was unable to cause flocculation of lysolecithin sols with either potassium or calcium chlorides. This difference is probably because lysolecithin is very soluble in water and consequently would require a very high concentration of salt to cause flocculation.

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SOME FACTORS AFFECTING THE R_F VALUES OF SYMPATHOMIMETIC CATECHOLAMINES

By D. J. Roberts*

From the Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W.3

Received March 25, 1963

The behaviour of three catecholamines on paper chromatograms is reported as an investigation of the effects of variables on R_F values. The use of these values as a means of locating and identifying catecholamines on paper is misleading as they are dependent on too many different factors.

Paper chromatography has been extensively used as a means of separating catecholamines in biological tissues and fluids, but the use of R_F values as an aid to identification is incompatible with the differing values reported for individual catecholamines. The chromatographic behaviour of some catecholamines has been examined, using a single solvent, to seek an explanation of the differences in values.

Methods

Whatman No. 1 papers were washed with hydrochloric acid (0·01N) by continuous descending flow for 20 hr., dried and cut. Circles of 1 cm. diameter, placed 3 cm. apart, were marked out 5 cm. from the bottom edge of the papers which were then fashioned into cylindrical form (Wolfson, Cohn and Devaney, 1949), and solutions of usually 25 μg. of each amine in 0·01 ml. distilled water were applied within the marked areas. The papers were placed into tanks (55 cm. high, 14 cm. diameter) containing 180 ml. of phenol containing 15 per cent v/v 0·01N hydrochloric acid, equilibrated with the internal atmosphere for not less than 24 hr. at room temperature. Chromatography was at 22–29° by the ascending technique for either 16 to 22 or 40 to 46 hr., the solvent fronts advancing 20 to 30 cm. and 35 to 44 cm. respectively. After removing the solvent by washing with benzene, and drying, the spots were located by spraying the papers with a solution of potassium ferricyanide (0·44 g.) in sodium hydroxide (100 ml. 0·05N). The R_F values were measured from the centre of each spot.

Drugs. (−)-Noradrenaline acid tartrate (L. Light & Co. Ltd.), (−)-adrenaline acid tartrate (Burroughs Wellcome & Co.) and (±)-isoprenaline sulphate (Burroughs Wellcome & Co.) were obtained commercially.

Results

As expected R_F values were found to depend on the grade of paper (Table I) and temperature (Table II). 2·5 μg. of adrenaline or noradrenaline, and 1·0 μg. of isoprenaline were the minimum amounts required to

* Present address: School of Pharmacy, Brighton College of Technology, Moulsecoomb, Brighton, 7.

obtain visible spots on oxidation. Although the use of more than 200 μg. of each amine caused streaking and tailing, the volume applied was without effect as shown by the identical R_F values obtained from seven different dilutions each containing 25 μg. of the amines (0·005 ml. to 0·5 ml.).

TABLE I

R_F VALUES OF REFERENCE AMINES COMPARED SIMULTANEOUSLY ON TWO DIFFERENT MEDIUM FLOW GRADES OF PAPER

Solvent, phenol containing 15 per cent v/v 0·1N HCl. Temperature 22 to 25°. Solvent front 25 to 30 cm. above point of application

Paper No.		Catecholamine R_F values		
		Noradrenaline	Adrenaline	Isoprenaline
Whatman No. 1	Range over 13 observations	0·09–0·14	0·34–0·43	0·54–0·64
	Mean with standard error	0·12 (±0·006)	0·38 (±0·007)	0·58 (±0·007)
Whatman No. 40	Range over 13 observations	0·055–0·084	0·23–0·32	0·44–0·55
	Mean with standard error	0·068 (±0·002)	0·29 (±0·012)	0·51 (±0·01)

With papers previously washed with hydrochloric acid (0·01N) compact spots were obtained from all three amines, but when the acid washing process was omitted, tailing of the isoprenaline and, to a lesser extent, the adrenaline spots was observed, while noradrenaline showed complete separation into two spots. That the additional spot was derived from noradrenaline was proved by chromatographing the amines individually.

TABLE II

R_F VALUES OF REFERENCE AMINES CHROMATOGRAPHED AT DIFFERENT TEMPERATURES

Solvent, phenol containing 15 per cent v/v 0·1N HCl. Solvent front 18 to 22 cm. above point of application

Catecholamine	Temperature °C		
	11	23	40
Isoprenaline	0·55 0·53	0·60 0·61	0·72 0·73
Adrenaline	0·36 0·35	0·41 0·41	0·52 0·53
Noradrenaline	0·13 0·12	0·14 0·15	0·25 0·25

In addition, the R_F values of each of the amines were markedly reduced on papers not washed with acid. The effects of washing with acid were reproduced when the papers were sprayed with hydrochloric acid (0·01N), but not when they were sprayed with distilled water. In all instances the right half of the paper only was sprayed, the remainder serving as control. On chromatographing the amines (25 μg.) from hydrochloric acid (0·01N) solution (0·025 ml.) on papers half-sprayed with acid as above, noradrenaline produced only a single spot on both the untreated and acid-treated paper but the tailing of the isoprenaline and adrenaline spots and the lower R_F values on the unsprayed half were still evident.

When the amines were applied at 5 cm. intervals from the bottom edge of the paper the results indicated that R_F values increased with the distance of the solvent front above the application point or decreased with the distance of this point from the solvent surface (Fig. 1). Further experiments in which the distance of the solvent front above a fixed application point was varied showed that R_F values were lower the further the solvent front advanced. Fig. 1 also shows that on one occasion this relationship was not true for the application point nearest the solvent surface.

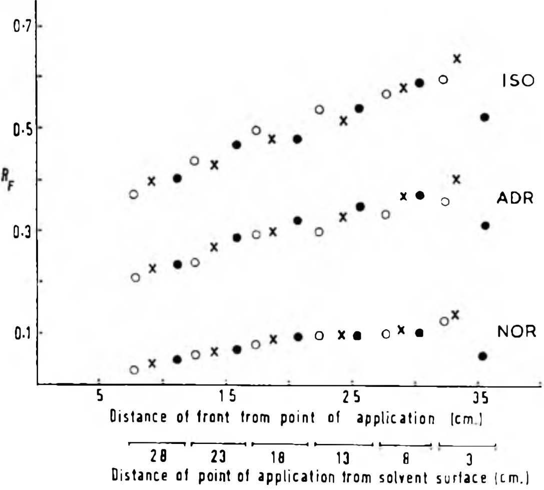

FIG. 1. R_F values of noradrenaline (NOR), adrenaline (ADR) and isoprenaline (ISO) developed in phenol containing 15 per cent v/v 0·1 N HCl when applied from distilled water at varying distances from the solvent surface. Open circles, crosses and closed circles represent three different experiments.

Successive running of chromatograms in any one tank resulted in a fall in R_F values, the effect appeared to be dependent on the number of times the solvent was used and was intensified by omission of the equilibration process or by gassing the tanks with carbon dioxide before use. In the absence of equilibration, prespraying the papers with distilled water and allowing them to dry at room temperature resulted in significant (but smaller than after acid treatment) increases in R_F values an effect that was completely inhibited by drying the papers at 40°. The R_F values on acid-sprayed papers remained high after drying at 40°. A return of R_F values to levels slightly higher than originally obtained was observed after beakers of distilled water or hydrochloric acid (250 ml. 0·01N) had been placed in the centre of the solvent and the internal atmosphere of the tank allowed to equilibrate for 12 hr. When some of the previous experiments were repeated in the presence of this excess of aqueous phase in a tank of fresh solvent, the results were qualitatively the same as before, but the R_F values were higher and showed good stability with time. The R_F values of the amines when applied close to the solvent

surface, however, were always lower than expected when curves similar to Fig. 1 were plotted.

Aqueous solutions of the amines were also chromatographed using distilled water as the developing solvent. The papers were rapidly dried at 40° before location of the spots to prevent excessive diffusion, and although no separation was evident the amines appeared as streaks in the bulk of the paper.

Discussion

It is now accepted that simple partition between a stationary aqueous phase supported by the paper and a mobile organic phase is not the sole phenomenon involved in the separation of substances by paper chromatography. The results obtained using distilled water as the developing solvent and the demonstration that R_F values are dependent upon the grade of paper used indicate that adsorptive forces are important and that the "water cellulose complex" (Haynes and Isherwood, 1949; Martin, 1950), and hence the distribution isotherm, varies from paper to paper.

In general, R_F values fall with decreasing water content when miscible pairs of liquids are used as chromatographic solvents. In the phenol-hydrochloric acid system used for this study, preferential absorption of water results in the solvent becoming more "phenolic" as it flows up the paper. This phenomenon of "frontal analysis", previously noted in the collidine-water system by Kowkabany and Cassidy (1952), together with the change in the water content of the solvent as each successive chromatogram is taken out of the tank, offers an explanation of why the R_F values obtained vary inversely with the distance the solvent travels and with the number of times the solvent is used.

In the absence of prior saturation or equilibration of the paper, conditions required for "real partition chromatography" (Tscheshe, Grimmer and Seehofer, 1953), the stationary phase is taken either directly from the developing solvent or from its vapour during chromatography. The marked influence on R_F values of decreasing the water content of the vapour by gassing with carbon dioxide and of increasing the water content of the vapour by using beakers of water or acid, or increasing the temperature, infers that the vapour is a major source of the stationary phase; but the aqueous content of the vapour is dependent on the proportion of water in the liquid solvent. The dimensions of the tank, together with the small surface area of the developing solvent, makes it doubtful whether true equilibrium conditions are achieved throughout the vapour phase, and the lowering of R_F values with increased distance of solvent flow could be the result of increased evaporation of water from the higher areas of paper, where the vapour phase is not saturated as it would be near the solvent surface. The hypotheses of a concentration gradient for the water content of the vapour phase inside the tank and of "frontal analysis" are both supported by the fact that the higher the R_F value of the amine the steeper the slope of the linear curve of R_F value versus the distance travelled by the solvent (Fig. 1). Such a relationship might not be expected to hold true for an application point near the solvent

surface, where oversaturation of the paper with aqueous phase could result in interference with partition due to the marked water solubility of the amine salts.

Production of a bound stationary aqueous phase by spraying the paper with distilled water again results in higher R_F values, but the increased migration observed on acid-treated papers and their stability after procedures sufficient to drive off any bound water require further explanation.

The lack of demonstrable differences in the chromatographic behaviour of the amines between acid-washed and acid-sprayed papers means that it is not the removal by washing of traces of metal ions, responsible for retardation and tailing of spots (Haynes and Isherwood, 1949), that gives rise to increased R_F values. Alteration of the physical properties of the paper (increase in capillary size, reduction in adsorption) by the acid, and suppression of the ion-exchange properties of the free carboxylic groups of the carbohydrate network could be responsible for the beneficial effects of pretreating the paper with acid, but probably more than one factor is involved and more work is needed to elucidate the problem.

The phenomenon of multiple spot formation shown by noradrenaline on untreated papers may be explained in terms of the arguments presented by Beckett, Beaven and Robinson (1960) in their report on the effects of acids, other than those present in the developing solvent, on the formation of amine spots. The hydrochloric acid present in the developing solvent converts some of the noradrenaline acid tartrate, when applied from distilled water, to noradrenaline hydrochloride. Two spots are then formed on development; the lower one being noradrenaline associated with tartaric acid, and the upper one noradrenaline associated with hydrochloric acid.

The fact that chromatography of the same amounts of amine, from a concentration of hydrochloric acid sufficient to convert all of the noradrenaline to the hydrochloride salt, results in the formation by noradrenaline of a single spot, of the higher R_F value, lends support to the above suggestions.

The R_F values of adrenaline and isoprenaline must differ sufficiently from those of tartaric, hydrochloric and sulphuric acid to be uninfluenced by them.

Furthermore, since application of the amines from an excess of hydrochloric acid still resulted in lower R_F values on the untreated paper, conversion of the amines to hydrochloride salts is also eliminated as a cause of the increased migration on the acid-treated paper.

The results described are subject to the limiting sensitivity of the spray reagent, but serve to demonstrate the variability of catecholamine R_F values.

Acknowledgement. This work was undertaken while the author was receiving an educational grant from the Pharmaceutical Society of Great Britain.

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EFFECT OF ULTRA-VIOLET IRRADIATION OF PHENYLEPHRINE SOLUTIONS

BY F. P. LUDUENA, ANN L. SNYDER, AND A. M. LANDS

From the Sterling-Winthrop Research Institute, Rensselaer, N.Y.

Received February 15, 1963

Solutions of phenylephrine in distilled water were irradiated with ultra-violet light for 3 hr. The irradiated solutions tested on perfused guinea-pig lungs had a bronchodilator potency greater than that of the original non-irradiated phenylephrine solution. The bioassay and photofluorometric determinations (Shore and Olin method) suggested that adrenaline had been formed from phenylephrine in the irradiated solution.

As early as 1931, Ewing, Blickensdorfer and McGuigan reported that the pressor activity of aqueous solutions of 1-(4-hydroxyphenyl)-2-methylaminoethanol (oxedrine, Sympatol) was increased by ultra-violet irradiation, whereas the same treatment reduced the pressor activity of adrenaline solutions and made solutions of ephedrine hypotensive. Inactivation of adrenaline by ultra-violet irradiation had been reported earlier that year by Verda, Keer and Burge (1931). Konzett and Weis (1938, 1939) confirmed the results obtained with adrenaline and by biological and chemical tests were able to confirm also the formation of adrenaline in irradiated solutions of oxedrine.

The increase in activity produced by irradiation suggested the possibility that oxedrine had been changed to adrenaline. After irradiation, the adrenaline solutions were discoloured and a similar discoloration was seen after irradiation of oxedrine. This suggested that catechol formation may be one of the intermediate steps in the process of discoloration of phenolic sympathomimetic amines solutions induced by ageing. This possibility is of interest since West and Whittet (1960) found that 10 per cent solutions of phenylephrine [1-(3-hydroxyphenyl)-2-methylaminoethanol] stored at room temperature in amber-coloured bottles "became yellow or pink within a few weeks of issue", even when they contained sodium metabisulphite (0·1–0·2 per cent). When pronounced discoloration occurred after storage in colourless bottles, from prolonged storage or after the addition of hydrogen peroxide to the solution, the loss in pharmacological activity was minimal (pressor effect on anaesthetised rats or cats). West and Whittet also found no direct relation between loss of activity and amount of discoloration.

That oxidation resulted in discoloration of the solutions suggested the possibility that adrenaline may have been formed as a first step in this process and that the small amounts formed could not have been detected in the presence of large amounts of phenylephrine by the bioassay method used. To test for the possible formation of adrenaline by oxidation, we subjected solutions of phenylephrine to ultra-violet irradiation and estimated the adrenaline content by bronchodilator activity and by chemical analysis.

Methods

Solutions of phenylephrine hydrochloride in distilled water (25–50 ml.) were placed in Petri dishes (3·5 in. diameter and 0·5 in. depth) on an adjustable platform under an ultra-violet lamp (Analytic Model Quartz Lamp, Engelhard Hanovia, Inc., Newark, N.J.). The solutions, placed approximately 23 cm. below the burner and 3·5 cm. below the Hanovia SC-5028 heat filter, were irradiated for 3 hr., after which they had the colour of a strong tea infusion. Distilled water was added to replace that lost by evaporation.

The solutions were tested on the perfused guinea-pig lung preparation (Sollmann and von Oettingen, 1928; Tainter, Pedden and James, 1934) with modifications which have been described previously (Luduena, von Euler, Tullar and Lands, 1957; Lands, Luduena, Hoppe and Oyen, 1958). In two experiments, bronchoconstriction was produced with histamine phosphate added to the Krebs-Henseleit solution (1 : 8 million, as base) in the reservoir. In the third experiment carbachol (1 : 10 million) was used instead of histamine.

The technique of Shore and Olin (1958) with the modifications described by Lund (1959) was used to estimate biochemically the concentration of catecholamines. Direct development of fluorescence in dilutions of the original solution after treatment with iodine was of questionable value because of the brown colour of the solution caused by irradiation. For this reason the phenylephrine solutions were extracted with sodium chloride-saturated butanol. The solvent phase was added to 2 volumes of heptane and then extracted with 0·01N hydrochloric acid. Aliquots of the acid phase were taken for fluorescence development by iodine oxidation. Fluorescence was developed at pH 3·0 and 5·0 to give a differential estimation of adrenaline and noradrenaline.

Results

Experiment I. A solution containing phenylephrine hydrochloride (1 mg./ml.) was irradiated for 3 hr. and then tested on two lungs. With one of the preparations, a dose of 0·025 ml. (25 μg. in terms of the original concentration of phenylephrine) produced bronchodilation comparable to that of 0·5 μg. of adrenaline (as base). In the other preparation the effect of 0·025 ml. was slightly less than that of 0·5 μg. of adrenaline. A 400 μg. dose of non-irradiated phenylephrine produced much less bronchodilation than 0·5 μg. of adrenaline. In terms of adrenaline, the bronchodilator effect of the irradiated solution corresponded to a concentration of approximately 20 μg./ml. or to the conversion of about 2·2 per cent of the original content of phenylephrine.

Experiment II. Two solutions were irradiated for 3 hr. One (A) contained 10 mg. and the other (B), 5 mg. of phenylephrine hydrochloride per ml. The solutions were kept in the refrigerator and tested the following day on the perfused lung preparation. Bronchodilatation was obtained with both solutions. On this preparation, a non-irradiated phenylephrine solution of 5 mg./ml. was approximately one-fourth as active as

solution B. Tested in comparison with adrenaline, 0·005 ml. of both solutions A and B produced approximately the same bronchodilator effect; a dose of 0·2 μg. of adrenaline was slightly more active.

From this it follows that if the activity were due to adrenaline alone, both solutions would contain 40 μg./ml. of adrenaline. But if no decomposition of the phenylephrine, other than that changed to adrenaline, had occurred, the adrenaline content would be 40 μg./ml. less the activity of the unchanged phenylephrine. This was found in a non-irradiated solution of 5 mg./ml. to be 1/4 of the activity of the irradiated solution of the same strength (solution B). Therefore the phenylephrine activity of solution B would be 1/4 of its total activity, i.e. 10 μg./ml. and its adrenaline content 30 μg./ml. As the phenylephrine activity in the 5 mg./ml. irradiated solution is equivalent to 10 μg. of adrenaline, that of solution, containing 10 mg./ml. phenylephrine, contributes 20 μg./ml. activity. Thus solution A would contain 20 μg./ml. of adrenaline. Obviously some decomposition of phenylephrine has occurred as shown by the changes in activity and the discoloration and therefore the adrenaline content would be between 20 and 40 μg./ml. for solution A and between 30 and 40 μg./ml. for solution B. These values were higher than those obtained by the fluorescence tests.

TABLE I

BRONCHODILATOR ACTIVITY OF ADRENALINE PHENYLEPHRINE AND IRRADIATED PHENYLEPHRINE SOLUTIONS (GUINEA-PIG LUNG)

Drugs*	Dose μg.*	Decrease in perfusion pressure (cm. H_2O)	
		Mean ± s.e.	Range
Adrenaline bitartrate	0·5	16·1 ± 2·6	5·0–27·5
	1·0	22·4 ± 2·1	12·0–28·5
	2·0	31·2 ± 1·8	25·5–39·0
	4·0	37·6 ± 2·0	26·0–48·5
Phenylephrine hydrochloride	165·0	5·2 ± 1·7	0·0–12·5
	330·0	11·1 ± 2·5	0·0–16·5
	660·0	15·1 ± 2·9	0·0–28·5
	1320·0	16·3 ± 3·8	0·0–26·5
Irradiated †phenylephrine hydrochloride	82·0	16·0 ± 2·7	4·0–29·0
	165·0	22·5 ± 2·3	7·0–29·5
	330·0	25·6 ± 1·6	17·5–33·5
	660·0	33·8 ± 1·7	25·0–41·0

* In terms of the bases.
† In terms of the original concentration of phenylephrine.

Non-irradiated phenylephrine solutions did not fluoresce when subjected to the iodine test procedure. The irradiated solutions (A and B) fluoresced at both pH 3 and 5 and gave values equivalent to an adrenaline content of 12–20 μg./ml. Noradrenaline shows little fluorescence at pH 3 (Shore and Olin, 1958; Lund, 1959) indicating that the catecholamine found was probably adrenaline because the irradiated solutions showed substantial activity when examined for bronchodilator action.

Experiment III. A solution (200 ml.) of phenylephrine hydrochloride (5 mg./ml.) in distilled water was prepared and samples irradiated as in the previous experiments (50 ml./Petri dish) for 3 hr.

The original non-irradiated solution, the irradiated solution, and a solution of adrenaline were tested on 10 guinea-pig lungs. Four graded doses of each solution were tested on each lung, the 12 doses being injected in a randomised sequence. This was important since in some preparations the sensitivity to the bronchodilators increased during the testing period. The tests were made over a period of several days. During this time, the phenylephrine solutions and a stock solution of adrenaline bitartrate (1 mg./ml. as base) were kept in closed containers in the refrigerator at approximately 4°.

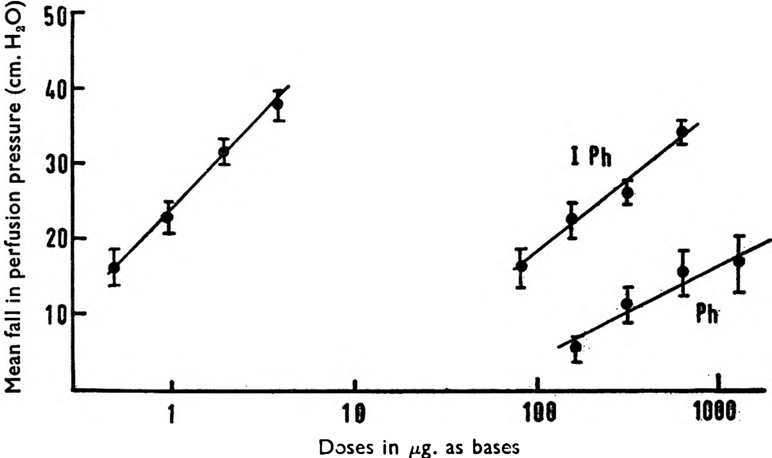

FIG. 1. Bronchodilator effect on perfused guinea-pig lung of adrenaline, phenylephrine (Ph) and irradiated phenylephrine (IPh).

At the dosage used, adrenaline and irradiated phenylephrine produced bronchodilation as measured by the fall of perfusion pressure. At the 165 and 320 µg. doses, phenylephrine produced no dilatation in 3 out of the 10 lungs, and 2 out of the 10 lungs for the respective doses. One of the preparations failed to respond to any of the 4 doses used.

The effects of the various doses on the perfusion pressure (mean ± s.e. and range) are presented in Table I. By plotting the mean response (fall of perfusion pressure) against the dose on semi-log paper approximate linear regression lines were obtained (Fig. 1).

The results show that ultra-violet irradiation produced an approximate ten-fold increase in bronchodilator activity of the phenylephrine solutions. Therefore, approximately nine-tenths of this activity is due to a compound or compounds formed during the period of irradiation. By comparing the highest and the lowest point of the dose-effect curve of irradiated phenylephrine (I.Ph) with the corresponding points in that of adrenaline, the latter (as base) was found to be 170 to 260 times more active. As each ml. of the original 5 mg./ml. solution contained phenylephrine base, 4·1 mg., the above activity ratios correspond to an adrenaline content of 24·4 to 15·4 µg./ml. This would be the amount of adrenaline if all the

phenylephrine had been destroyed but, if only a small amount had been destroyed by irradiation, that remaining would contribute approximately one-tenth of the activity of the irradiated solution. Then the content of adrenaline would be nine-tenths of the above values, i.e., 14 to 22 μg./ml. Obviously some decomposition occurred as indicated by the increase in biological activity and therefore the range of estimates of the adrenaline content should be extended to include maximal and minimal phenylephrine decomposition. This gives a range of 14 to 24·5 μg./ml. Four photofluorometric determinations of adrenaline made on different days gave a mean $\pm$ s.e. of 24·3 $\pm$ 3·8 μg./ml. This agrees, within the error of the methods, with the bioassay determination.

Discussion

The most likely explanation for the increase in bronchodilator activity of phenylephrine solutions after ultra-violet irradiation is that a small amount of adrenaline was formed. This would require the oxidation of the ring in the *p*-position. Other catecholamines such as dopamine and noradrenaline are bronchodilators, but their activity is less than that of adrenaline. The results of the fluorescence tests give adrenaline values which, considering the error of the methods, are not different from those obtained by estimating bronchodilator activity.

The investigation of Ewing and others (1931), Konzett and Weis (1938, 1939), Konzett (1941) and Holtz and Credner (1943) provided indirect evidence for the conversion of various monophenolic sympathomimetic amines into the corresponding catechol analogues. Oxedrine, tyramine, *p*- and *m*-oxyephedrine and *p*- and *m*-norsynephrine (Shepherd and West, 1952) solutions acquire higher pressor activity by irradiation. There is disagreement about the effect of ultra-violet irradiation on ephedrine solutions. According to Ewing and others (1931) irradiation reversed the pressor effect of ephedrine solutions (small doses of the catechol analogue of ephedrine lowers blood pressure, Schaumann, 1930; Tainter, 1933). However, Konzett (1941) reported that (−)- and (+)-ephedrine and (−)- and (+)-pseudoephedrine solutions retained their pressor effect even after long irradiation although discoloration occurred.

In all those investigations, increase in activity was accompanied by discoloration of the solutions, which apparently was the result of further oxidation of the catecholamine formed. This suggests that the corresponding catechol analogue is formed in solutions of mono-phenolic amines during the process of colour production by ageing.

Acknowledgements. We are indebted to Mr. J. P. McAuliff and Dr. A. Arnold for the fluorometric determinations of epinephrine.

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A NOTE ON THE ANTIFUNGAL ACTIVITY OF PENTACHLOROPHENYL DODECANOATE

By Ronald A. McAllister

From the University Department of Surgery, The Western Infirmary, Glasgow

Received February 4, 1963

The antifungal activity of pentachlorophenyl dodecanoate against certain dermatophytes has been studied. The substance, in Sabouraud's maltose agar in 2 per cent concentration, inhibited the growth of *Microsporum canis*, *Trichophyton interdigitale*, *Trichophyton rubrum*, and *Microsporum audouini*. No activity was observed against *Candida albicans*, and only a slight inhibition in the growth of *Epidermophyton floccosum*. A 3 per cent concentration of the ester in an ointment base effected complete inhibition of *Trichophyton interdigitale* and *Microsporum canis* alone, and in mixed cultures.

The fungistatic activity of fatty acids, has been shown by Kiesel (1913) to increase with the number of carbon atoms, up to 11 or 12, maximum activity being obtained with 10-undecanoic acid. As a result of more critical tests, Golden and Oster (1947) demonstrated that this activity was relatively weak. Attempts to increase this by the formation of esters such as propylene glycol dipropionate, and propylene glycol dipelargonate, were not successful, the latter giving 58 per cent of treatment failures in cases of tinea capitis (Sullivan and Bereston, 1952). Since trichlorophenol is a clinically effective fungicide in superficial mycoses, but causes skin irritation (Hopkins, Fisher, Hillegas, Ledin and Camp, 1946), it was considered that esters of the type, $Me{\cdot}[CH_2]_n{\cdot}COOR$ or, $CH_2:CH{\cdot}[CH_2]_n{\cdot}COOR$, where n is less than 10 and R a halogenated phenol, might show antifungal activity. Further, esterification in this manner might lessen the toxicity of the phenol, since the *ortho*-alkyl derivatives of *p*-chlorophenol show a decrease in toxicity from the methyl to the n-heptyl, and at the same time an increase in antibacterial activity as the molecular weight increases (Klarman, Shternov and Gates, 1934).

During preliminary work on compounds of the type postulated above, it was found that the pentachlorophenyl dodecanoate was manufactured in large amounts as a fungicide for the textile industry. The present paper is a preliminary study of the activity of this substance against certain dermatophytes.

Materials and Methods

According to the manufacturers, the pentachlorophenyl dodecanoate is normally prepared and used in a liquid form at room temperature. For the present investigation, a purer form was made to Ministry of Supply Specification C.S. 2616. This is a solid with a melting point of 46·5°, and a setting point of 33°. The white, wax-like solid is insoluble in water and the lower alcohols, solubility beginning to be appreciable from isopropanol upwards. It is soluble in non-polar solvents such as acetone, benzene, and methyl ethyl ketone.

Organisms Used

Cultures of *Trichophyton rubrum* D 361, *Microsporum canis* 352, *Trichophyton interdigitale* 296, and *Trichophyton mentagrophytes* 336, were obtained from the London School of Hygiene and Tropical Medicine. Other organisms were: *Microsporum audouini*, *Candida albicans*, and *Epidermophyton floccosum*, which had been isolated during routine work in a Hospital Bacteriology Department. These were maintained on Sabouraud's maltose agar, pH 5·2 and kept at 25°. Subcultures were usually made at 6 to 8 weeks. Following suggestions by Golden and Oster (1947) and McCrea (1940) 10 to 15 day cultures were used in the assays so that all growth elements such as hyphae, micro- and macronidia and chlamydospores were present.

Experimental and Results

The ester was incorporated in Sabouraud's maltose agar by melting 200 ml. of the medium in a Koch steam steriliser, and adding 2 g. of pentachlorophenyl dodecanoate in thin shavings. During the addition, the mixture was stirred mechanically under sterile conditions, then 10 ml. aliquots were dispensed into sterile Universal containers. Since the specific gravity of the ester is 1·25, slopes were rapidly cooled to prevent deposition, and stored at 6°.

In preliminary assays with *T. rubrum* D361, pieces of mycelia from 15 day cultures of the organism were inoculated into 6 tubes of Sabouraud's maltose agar. At the same time, 6 tubes of medium containing 1 per cent of the ester were inoculated in a similar manner. At this stage, no attempts were made to standardise the size of the inoculum, although in each case mycelial strands about 5 mm. long were used. The periphery of each was then roughly outlined on the outer wall of the container with Indian ink, and the tubes incubated at 25°.

The results showed that there was slight growth in the medium containing 1 per cent pentachlorophenyl dodecanoate, after 6 days at 25°, as opposed to 2 days with the controls. After 14 days, no further growth could be detected, and the cultures had a shrunken and granular appearance. When the concentration of the ester was increased to 2 per cent, the growth of *T. rubrum* was inhibited for 14 days, one tube out of six, showed slight growth, and then no further growth could be detected.

A 2 per cent concentration of pentachlorophenyl dodecanoate, under similar test conditions, was found to inhibit the growth of *M. canis*, *T. interdigitale* and *M. audouini*. No action was found in plates using *C. albicans*, and only a slight inhibitory effect with a strain of *E. floccosum*.

Antifungal Activity in an Ointment Base

An ointment base consisting of liquid paraffin, 12; white soft paraffin, 30; and cetomacrogol emulsifying wax, 18 g. was prepared and sterilised at 150° for 1 hr. and allowed to cool to about 70°. Sterile Sabouraud's broth (140 ml.) was then added aseptically with mechanical stirring. Just before solidification, the emulsion was poured into sterile Petri

plates, which served as controls. Test plates were prepared by taking a similar mixture, heating it to 70°, and adding 6 g. of pentachlorophenyl dodecanoate in thin shavings, and then adding 134 ml. of sterile Sabouraud's broth.

Inoculum. Cultures of *T. interdigitale* and *M. canis* on Sabouraud's maltose agar were inoculated into Sabouraud's broth and incubated at 25° for 10 days. The mycelial pads in each were broken up, and the tubes shaken mechanically. Test plates were inoculated by flooding with 2 ml. of the cellular suspension, and incubated at 25°. Control plates without the fungicide were similarly treated. Table I shows the growth of both organisms without the test substance.

TABLE I

GROWTH PATTERNS IN AN EMULSION MADE WITH SABOURAUD'S BROTH WITHOUT PENTACHLOROPHENYL DODECANOATE

Culture	Days after Inoculation	
	4	12
T. interdigitale plus *M. canis*	Growth. Surface hyphae	Profuse growth over plate. Pale yellow pigment present
M. canis	Rapid growth. Pale yellow pigment	Growth over whole plate. Yellow pigment
T. interdigitale	Patches of white mycelium	Growth over whole plate

With a concentration of 3 per cent pentachlorophenyl dodecanoate, there was complete inhibition of *T. interdigitale* and *M. canis* alone and in mixed culture.

Discussion

The difficulty of assaying water-insoluble compounds for antifungal activity has been noted by Golden and Oster (1947a) and by Reddish (1947). Golden and Oster (1947a) introduced an agar diffusion method, in which the substance under test is dissolved in ethanol and 1 ml. aliquots placed in cups cut in the culture plate. Although these authors found the method successful in assaying simple compounds such as phenol, benzoic acid and related products, in our hands the method was unsatisfactory. Pentachlorophenyl dodecanoate is not very soluble in ethanol, and the long incubation times necessary for growth of the cultures caused the solutions to evaporate.

The insolubility of the ester in water also caused difficulties when attempts were made to determine whether the action was fungistatic or fungicidal. After being in contact with the emulsion described previously, the pieces of mycelium, or mycelial pads cannot be washed free of the active material with Sabouraud's broth, before placing in fresh medium. For some types of compounds, Golden and Oster (1947b) used a 30 per cent v/v solution of acetone in water to effect removal, before subculturing. With pentachlorophenyl dodecanoate the procedure is not applicable.

Acknowledgements. I wish to thank Professor Sir Charles Illingworth for his encouragement, and offer of facilities in his Department for

carrying out this work. Acknowledgements are also made to the Institute of Medical Laboratory Technology for a Grant, and the Directors of Catomance Ltd. for their help in arranging supplies of the purified material.

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THE SYNTHESIS OF *N*-SUBSTITUTED AMIDINES OF POTENTIAL PHARMACOLOGICAL ACTIVITY

By J. A. Smith and H. Taylor

From the Department of Pharmacy, Institute of Technology, Bradford

Received February 27, 1963

A series of *N*-3-diethylaminopropyl benzamidines has been prepared. The method used was the well-known Pinner synthesis (Pinner, 1892) of the imido-ester hydrochloride and then further reaction with 3-diethylaminopropylamine monohydrochloride. The compounds were tested for histamine-like, antihistamine and adrenergic neurone blocking activity. Some nonspecific antihistamine activity was found; there was no other activity.

Although the structure of *N*-dialkylaminopropyl amidines II bears a formal similarity to that of histamine I, little work appears to have been done in investigating the possibility of these compounds having histamine-like or antihistamine activity.

NH
HC CH
N—C—$CH_2 \cdot CH_2 \cdot NH_2$
I

NH
R-C
$NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NR_2$
II

Certain *N*-dialkylaminoalkyl benzamidines have already been prepared as antimalarials (Curd and Raison, 1947), as antibacterials (Fullar, Tonkin and Walker, 1945), and also as antituberculosis agents (Charlton, Maliphant, Oxley and Peak, 1951), but no reference was made to any possible antihistamine properties of these compounds.

No attempt, however, appears to have been made to prepare a series of *N*-substituted amidines with a constant *N*-dialkylaminopropyl side chain. Therefore, it was decided to prepare such a series (Table I), varying the aromatic characteristic R in an attempt to find whether this related structure had any antihistamine activity.

Experimental

The method used was the Pinner synthesis (Pinner, 1892) of the imido-ester hydrochlorides which were converted into the corresponding amidines by treatment with 3-diethylaminopropylamine monohydrochloride. In this case, the reaction between the imido-ester hydrochloride and the 3-diethylaminopropylamine base as described by Curd and Raison (1947) and by Fuller and others (1945) resulted in the decomposition of the imido-ester hydrochloride, with the evolution of ammonia. It was found that on the addition of 3-diethylaminopropylamine monohydrochloride, no ammonia was evolved and the reaction proceeded to give good yields of the *N*-diethylaminopropyl benzamidines. The methods described below apply generally.

TABLE I

$$R-C(=NH)-NH\cdot CH_2\cdot CH_2\cdot CH_2\cdot N\cdot Et_2$$

No.	R	Salt	m.p. °C	Formula	Found per cent				Required per cent				Found equiv. wt.	Required equiv. wt.
					C	H	N	Cl	C	H	N	Cl		
1a	C_6H_5—	di-HCl	163–4	$C_{14}H_{25}Cl_2N_3$	55·2	8·3	13·7	22·9	54·9	8·2	13·7	23·2	153	153
1b	C_6H_5—	dipicrate	166–7	$C_{20}H_{27}N_6O_7$	44·9	4·1	18·1	—	45·2	4·2	18·2	—	344	346
2a	*p*-Cl—C_6H_4—	di-HCl	237–9	$C_{14}H_{24}Cl_3N_3$	49·2	7·0	12·0	31·2	49·3	7·1	12·3	31·3	170	170
2b	*p*-Cl—C_6H_4—	dipicrate	193–4	$C_{20}H_{26}ClN_6O_7$	43·2	4·2	17·3	5·1	43·0	3·9	17·4	4·9	362	363
3a	*p*-MeO—C_6H_4—	di-HCl	204–5	$C_{15}H_{27}Cl_2N_3O$	53·2	8·1	12·4	21·0	53·6	8·1	12·5	21·1	169	168
3b	*p*-MeO—C_6H_4—	dipicrate	149–51	$C_{21}H_{20}N_6O_8$	44·9	4·4	17·3	—	44·9	4·3	17·5	—	364	361
4a	*p*-Me—C_6H_4—	di-HCl	195–7	$C_{15}H_{27}Cl_2N_3$	55·9	8·5	13·4	22·1	56·2	8·5	13·1	22·1	160	160
4b	*p*-Me—C_6H_4—	dipicrate	137–8	$C_{21}H_{29}N_6O_7$	45·5	4·2	17·8	—	45·9	4·4	17·9	—	352	353
5a	*p*-Br—C_6H_4	di-HCl	215–7	$C_{14}H_{24}BrCl_2N_3$	43·3	6·4	11·0	18·5	43·6	6·2	10·9	18·4	192	193
5b	*p*-Br—C_6H_4—	dipicrate	195–6	$C_{20}H_{26}BrN_6O_7$	40·4	4·1	16·0	—	40·5	3·6	16·3	—	385	385

The equivalent weights of the dihydrochlorides were determined by titration with 0·1N silver nitrate solution.
The equivalent weights of the dipicrates were determined by titration with 0·1N perchloric acid in glacial acetic acid.

Preparation of ethyl benzimidate hydrochloride. A solution of dry benzonitrile (10·3 g. 0·1 mole) and "super-dry" ethanol (13·8 g. 0·3 mole) in dry chloroform (30 ml.) was saturated with dry hydrogen chloride gas at 0°. The mixture was allowed to stand for four days at room temperature and the solvent removed under reduced pressure. The residue was dried quickly at 80° and the last traces of hydrogen chloride removed in a desiccator containing sodium hydroxide pellets to give ethyl benzimidate hydrochloride (17·25 g.) m.p. 119–120°. Yield, 93·0 per cent.

Preparation of N-*diethylaminopropyl benzamidine dihydrochloride.* A solution of ethyl benzimidate hydrochloride (9·275 g. 0·05 mole), 3-diethylaminopropylamine (6·83 g. 0·053 mole) and ethanolic hydrogen chloride (9·72 ml. of 5·4N solution 0·053 mole) in 20 ml. absolute ethanol was warmed at 40° for 8 hr. The mixture was allowed to stand overnight at room temperature, filtered and the solvent removed under reduced pressure. The residue was treated with sodium hydroxide solution (85 ml. of a 5 per cent solution) and the liberated oil taken up in ether. The ethereal solution was shaken with acetic acid (125 ml. of a 5 per cent solution), and the acid layer separated. Sodium hydroxide (85 ml. of a 5 per cent solution) was added to liberate the base which was again taken up in ether. The ether layer was separated, washed with water and dried ($MgSO_4$). The solvent was removed, ethanolic hydrogen chloride (18·52 ml. of 5·4N solution), isopropanol and then ether were added to give N-*diethylaminopropylbenzamidine dihydrochloride* (10·2 g.). The yield was 66·7 per cent. On recrystallisation from isopropanol: ether, white crystals were obtained, m.p. 163–164°.

PHARMACOLOGY

The amidine dihydrochlorides were tested on guinea-pig ileum for histamine or antihistamine activity. Although compounds 2a and 5a (20 μg./20 ml. bath) reduced the response to 0·1 μg. histamine by about half, the same dose reduced the response to acetylcholine, 5-hydroxytryptamine and barium ions by a similar amount. Compound 3a also had slight spasmolytic activity but 1a and 4a showed no blocking action.

It was reported by Boura, Copp and Green (1962) that certain acetamidine derivatives have an adrenergic neurone blocking action. Our compounds structurely resembled some of these compounds and therefore, 2a and 4a were tested but were found to have no such activity.

DISCUSSION

Although compounds 2a and 5a were found to be antagonistic to histamine, the action is nonspecific, since they also antagonise acetylcholine, 5-hydroxytryptamine and barium ions. This is not unexpected since most specific antihistamines have two aromatic ring structures.

It appears that the *para*-halogen substituent, and to a lesser extent the methoxy group, is necessary for activity in this limited series, since the unsubstituted and the *para*-methyl substituted derivatives have no activity.

This is interesting because many antihistamines have *para*-halogen and methoxy substituents.

Acknowledgements. We wish to thank the Department of Pharmacology, Institute of Technology, Bradford, and the Wellcome Research Laboratories, Beckenham, for their assistance in the testing of these compounds.

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A NOTE ON *PLANTAGO MAJOR* SEEDS: A SUBSTITUTE FOR ISPAGHULA

By S. M. J. S. Qadry

From the Pharmacognosy Laboratory, Institute of History of Medicine and Medical Research, Delhi-6, India

Received January 28, 1963

The pharmacognostical features of the seeds of *Plantago major*, a substitute of "Ispaghula" (*P. ovata*) are described and illustrated, and points of difference indicated.

THE seeds of *Plantago major* Linn. known as "Lahuriya" in Hindi and "Bartang" in Persian, are used for a variety of ailments and have been claimed by various authors on indigenous drugs (Kritikar and Basu, 1933; Chopra, Handa and Kapur, 1958; Mukerji, 1953; Khan, 1917) to be good substitute for *Plantago ovata* seeds (ispaghula), which are considered as a remedy for dysentery and chronic diarrhoea and are official in Indian Pharmacopoeia (1955) and British Pharmaceutical Codex (1959).

Controversy has existed about the botanical origin of *P. major* seeds. Some of the earlier workers attributed the seeds to *P. psyllium* and *P. lanceolata*. This confusion was rectified by Skyrme (1935) who germinated the seeds and identified the grown plants. This was confirmation of the observation of Thiselton-Dyer (1884), who identified the plants grown at Kew, from the seeds, as *P. major*. Further confirmation has been obtained in this work by comparison of commercial samples from a number of sources with the seeds of *P. major* plants, growing wild at Mussoorie and in Kashmir, which have been identified. The seeds agreed both in morphological and anatomical characters.

Further, it has been found that the seeds of *P. major* possess much less mucilage than those of *P. ovata* and hence claims of usefulness do not seem to be well founded. However, Wasicky (1961) reported that the Brazilian *P. major* var. *Cruenta* swelled the most in a comparison with *P. ovata* (= *P. ispaghula* Roxb.) and *P. psyllium* and hence to be most effective as laxative.

The seeds of *P. major* are reported to contain (Wehmer, 1931) 18·8 per cent crude protein, 19 per cent crude fibre, 10–20 per cent fatty oil, 8·25 per cent water and 5 per cent ash. Ogata and Nishioja (1924) isolated from the seeds not only a glucoside but also choline and succinic and platenollic acids.

The pharmacognosy of *P. ovata*, *P. psyllium* and *P. indica* is already known (Trease, 1958; Wallis, 1960; and others). However, no work on *P. major* which is commonly used in India and elsewhere has been reported.

Material and Methods

Samples of the seeds were obtained from different drug dealers in India and also from plants growing wild in Kashmir and Mussoorie.

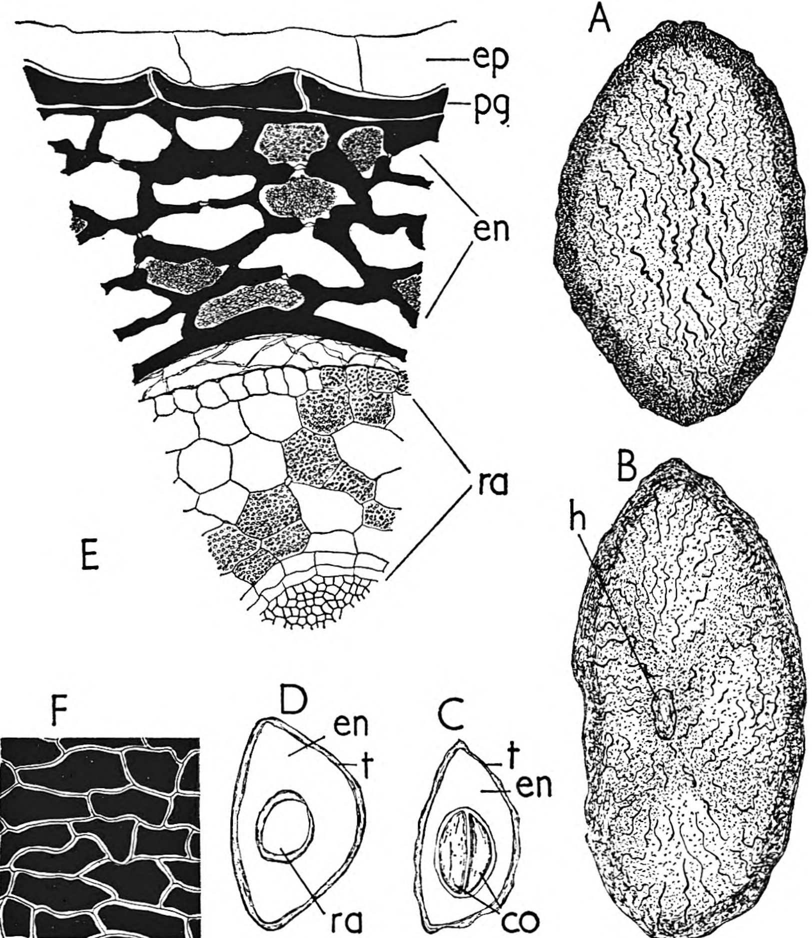

FIG. 1. Seed of *Plantago ovata:* A, dorsal view × 50. B, ventral view × 100. C, cut in middle × 25. D, cut at one end × 25. E, transverse section × 400. F, pigment layer in surface view × 150. co, cotyledons; en, endosperm; ep, epidermis; h, hilium; pg, pigment layer; ra, radicle; t, testa.

Usual methods of sectioning and staining were employed. The methods used for determination of ash contents and swelling factor were those of Indian Pharmacopoeia (1955).

The plant (Fitch and Smith, 1949; Sowerby, 1867), a native of Europe and introduced into India is found growing in the temperate Himalaya from Kashmir to Bhutan at altitudes of 5–2,300 m., in western Tibet at 29–3,600 m., China, in Khasi hills at 12–1,400 m., Aka hills, Konkan, Mahableshwar and Poona in the Deccan. It is a small perennial herb, up to 37 cm. high with rootstock short, truntate; leaves all radical, alternate, subacute, or obtuse, base tapering and decurrent into the long sheathing petiole, oblong or oblong-ovate, sub-entire or toothed;

scape short; spike very long and slender, lax, cylindrical; flowers small, green, ovary two celled, four to eight seeded; seeds minute, angled, dull black, rugulose.

Macroscopy

The seeds are minute and variable in shape and size, being 0·8–1·5 mm. long and 0·48–0·8 mm. broad. They vary in colour from light brown to dark brown or almost black. The seeds exhibit a variable outline. They are ovate, or ovate with obliquely truncated apex or irregular in outline (Fig. 1A). This variation may be accounted for by the compact arrangement of 8 to 16 seeds in each capsule. The seeds are more or less planoconvex. In the centre of the ventral or the plane surface (Fig. 1B) there is a depressed oval area on one side of which the hilum is located as a light coloured dot. The surface of the seed shows beautiful ripple-like markings of a darker colour (Fig. 1A,B). These markings are wavy elevated ridges on the surface and their arrangement differs on the two surfaces. On the dorsal surface the ridges run in the direction of the longer axis of the seed (Fig. 2A). On the ventral surface they radiate outward from the hilar depression (Fig. 1B). On soaking in water, the seed coat swells and the seeds become enveloped with a colourless mucilage. The seeds possess a slightly bitter and oily taste. The mucilaginous taste is not well marked. The commercial samples show much debris which includes dehisced capsules and dissepiments.

TABLE I

FEATURES DISTINGUISHING *Plantago major* FROM ISPAGHULA

	Plantago major	*Plantago ovata*
Colour	Light brown to dark brown or almost black.	Dull; pinkish grey-brown.
Shape	Irregular in outline; ovate or ovate with obliquely truncated apex.	Boat-shaped; outline ovate
Dorsal surface	Convex with beautiful ripple-like markings of a darker colour.	Convex with a small elliptical or elongated shining reddish-brown spot.
Ventral surface	Almost plain, with depressed oval area in the centre, and with ripple-like markings of a darker colour all over.	Concave with a deep furrow, not quite reaching either end of the seed.
Size—		
Length	0·8–1·5 mm.	2·0–3·3 mm.
Breadth	0·42–0·8 mm.	1–1·6 mm.
Taste	Slightly bitter and oily	Mucilagenous
Weight of 100 seeds	0·0202–0·024 g.	0·15–0·19 g.
Swelling factor	4·37–5	10·25–13·50
Ash	Not more than 4·8 per cent	Not more than 3 per cent
Acid-insoluble ash	Not more than 0·8 per cent	Not more than 0·6 per cent

Microscopy

The testa is composed of 2 layers of cells (Fig. 1E).

The epidermis. This is a single layer of translucent thin-walled cells wherein the mucilage is found. The cells are rectangular, being elongated in the tangential direction (Fig. 1E). The mucilage takes a red colour with safranin; no lamellate appearance is observed.

Pigment layer. This layer is also composed of rectangular cells, the outer tangential walls of which bulge slightly to the inside. The radial

walls thus project outward prominently (Fig. 1E). The cells are filled with brownish pigment and measure T, 35—**44**—66 μ and R, 12—**14**—18 μ. In surface view, the pigment layer cells are irregularly rectangular to polygonal in outline (Fig. 1F) and measure 17—**55**—90 μ in diameter.

Endosperm. This forms the major part of the seed and completely surrounds the embryo (Fig. 1C,D). The cells show highly thickened cellulosic walls having pores for communication between the adjacent cells (Fig. 2E). The cell lumen is filled with fixed oil and protein. The cells measure 35—**44**—54 μ in diameter.

Embryo. The embryo is straight and lies in the middle along the long axis of the seed. The radicle is circular in transverse section (Fig. 1D) while the two cotyledons are planoconvex (Fig. 1C). The cells are parenchymatous and measure 16—**20**—28 μ in diameter. They are packed with aleurone grains.

One g. of *Plantago major* seeds when subjected to the absorbency test of the B.P.C. 1949 gave volume of not more than 5 ml. The seeds yielded not more than 4·8 per cent of total ash and not more than 0·8 per cent of acid-insoluble ash.

Points of distinction from isphagula are summarised in Table I.

Acknowledgement. The author is indebted to Hakim Abdul Hameed, President, I.H.M.M.R., for providing facilities for the present work and to his colleagues, Dr. M. A. Aziz, Dr. H. H. Siddiqui and Mr. M. Rizwan for their interest. Thanks are also due to Sir George Taylor, Director, Royal Botanic Gardens, Kew, for providing clues to references and Mrs. Sughra J. Qadry for the drawings.

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LETTERS TO THE EDITOR

A Biochemical Distinction Between Non-Steroid Anti-inflammatory and Analgesic Drugs

SIR,—As alternatives to steroids, phenylbutazone (Butazolidine) and acetylsalicylic acid are prescribed extensively for the management of rheumatic diseases. It is well known that these two drugs are moderately potent analgesics and exhibit antipyretic activity. These facts have lent credence to a belief that anti-inflammatory (potential antirheumatic) activity amongst non-steroid drugs is either synonymous with, or largely overlaps, analgesic activity in non-narcotic drugs. This belief has been strengthened by recent reports that mefenamic acid possesses antipyretic, antinociceptive and anti-inflammatory activity in animals (Winder, Wax, Scotti, Scherrer, Jones and Short, 1962) and is a promising analgesic for clinical use (Cass and Frederick, 1963).

TABLE I

SOME PROPERTIES OF NON-NARCOTIC ANALGESICS

DATA FOR ANTI-INFLAMMATORY ACTIVITY WERE DRAWN FROM PUBLISHED REPORTS (see text)

Analgesic	Anti-inflammatory activity	Effect on	
		Oxidative phosphorylation (liver)	Phosphate metabolism (cartilage)
Phenylbutazone	†	†	†
Oxyphenbutazone	†	†	†
Amidopyrine	†	—	—
Antipyrine	—	—	—
Paracetamol	—	—	—
Acetanilide	—	—	—
Acetophenetidine	—	—	—
Salicylic acid	†	†	†
Salicylamide	—	—	—
2 and 4-Hydroxyisophthalic acids ..	—	—	—
Mefenamic acid	†	†	†
3-Hydroxycinchophene	†	†	†

† Indicates activity.

Adams (1960) suggested that it was not justifiable to classify all analgesic-antipyretic drugs as a single group. He presented pharmacological data which indicated that non-narcotic analgesics could be divided into two groups: (i) those including salicylic acid, aspirin and phenylbutazone which suppressed inflammation in laboratory animals and (ii) other analgesics including several derivatives of salicylic acid, which were inactive in an anti-inflammatory test (ultra-violet-light induced erythema). It has now been found that this subdivision of the non-narcotic analgesics into at least two groups according to their anti-inflammatory properties, is wholly supported by biochemical data.

Oxidative phosphorylation in isolated liver mitochondria is uncoupled by non-steroid anti-inflammatory drugs (Adams and Cobb, 1958; Whitehouse and Haslam, 1962) and certain anti-inflammatory steroids (Gómez-Puyou, Pĕna-Díaz, Guzman-García and Laguna, 1963). There is evidence that these compounds will also uncouple oxidative phosphorylation in extrahepatic tissues, notably in connective tissues (Whitehouse and Boström, 1962; Whitehouse and Haslam, 1962). Table I correlates the anti-inflammatory activity of some

non-narcotic analgesics (Adams, 1960; Cutting, 1962; Winder and others, 1962) with their effects upon (a) oxidative phosphorylation in respiring liver mitochondria with succinate as substrate and (b) upon phosphate metabolism in a connective tissue (cartilage), determined *in vitro* according to procedures described elsewhere (Whitehouse and Haslam, 1962). A compound was considered to have no significant effect upon oxidative phosphorylation or phosphate metabolism if the P/O quotient, or phosphate incorporation by cartilage slices, in the presence of the drug (2mM) was not less than 85 per cent of the value obtained in parallel incubations without added drugs. These results confirm and extend Brody's (1956) observations of the effect of certain analgesics on oxidative phosphorylation.

The only compound which failed to uncouple oxidative phosphorylation *in vitro* but which is known to have some anti-inflammatory activity *in vivo*, was amidopyrine. Two of its metabolites, 4-aminoantipyrine and 4-*N*-acetylaminopyrine (Brodie and Axelrod, 1950; Halberkann and Fretwurst, 1950), were also tested and found to be as inactive *in vitro* as amidopyrine. Amidopyrine is approximately one-eighth as potent as phenylbutazone in the guinea-pig ultraviolet light erythema assay for anti-inflammatory activity (Adams, 1960).

Several other analgesics were tested and found not to uncouple oxidative phosphorylation at a concentration of 2mM. These included salicoylpiperidine (Profft and Hogel, 1962) phenyramidol hydrochloride, morphine sulphate, pethidine (meperidine) hydrochloride and carisoprodol: none of these is known to exhibit significant anti-inflammatory activity in laboratory animals.

On the other hand, sodium aurothiomalate (Myochrysin) and gold sodium thiosulphate (Sanochrysin) which are not analgesics but are sometimes prescribed as antirheumatic drugs, uncouple oxidative phosphorylation in liver mitochondria at concentrations of 3mM and 0·3mM respectively. Their gold-less chemical analogues, sodium thiomalate and sodium thiosulphate had no effect on oxidative phosphorylation at 5mM concentration. Antimalarial aminoquinolines such as chloroquine and hydroxychloroquine which manifest antirheumatic activity only after prolonged administration, could be distinguished biochemically from other non-steroid anti-inflammatory drugs by their failure to uncouple oxidative phosphorylation at concentration up to 5mM, even after pre-incubation with liver mitochondria for 4 hr. at 2°.

Department of Biochemistry,
University of Oxford.
South Parks Road,
Oxford.
M. W. WHITEHOUSE

June 19, 1963

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LETTERS TO THE EDITOR

The Sporicidal Activity of Phenol

SIR,—Sykes (1958), Bennett (1959) and Cook (1960) have adequately reviewed research on the antibacterial activity of phenolics. Cook (1960) has drawn attention to the lack of published work on the sporicidal efficiency of phenol, and has stated ". . . it has been suggested that some bacterial spores will survive for long periods in 5 per cent phenol, but all attempts by the author to isolate such a spore have so far proved unsuccessful".

We have been engaged in research into the sporicidal activity in aqueous medium of phenol against *Bacillus subtilis* spores prepared as previously described by Gilbert and Russell (1963).

Spores were treated with 5 per cent w/v phenol for 5 days at 37°, and the number of survivors was determined by plating samples into nutrient agar (Oxoid) at pH 7·4, after diluting the phenol well below its growth inhibitory concentration. Survivor counts were below 10 per cent of the control (phenol absent). This apparent sporicidal effect was less marked when the phenol concentration was reduced to 2·5 per cent w/v.

Two stages can be distinguished in the germination of spores (Lund, 1962; Gould, Hitchens and Hurst, 1963): (i) initiation—the spores, called "bright" spores, lose their refractivity and heat resistance and stain readily: (ii) outgrowth—in which the spores swell and the vegetative cell is released. Recent work has shown that L- alanine and D- glucose stimulate spore germination (e.g. Powell, 1957) and it was thought that the addition of these substances at optimal concentrations to the recovery media might increase the number of survivors. This was found to be so and it thus seems probable that high concentrations of phenol can inhibit some stage or stages in the germination of spores of this organism. It is of interest to note that Lund (1962) has found that the initiation stage in various strains of *B. subtilis* was inhibited by phenol as distinct from cetrimide which allowed initiation to proceed but prevented outgrowth. Lund (1962) also showed that a 2·5 per cent w/v solution of phenol had some sporicidal effect at both 25° and 37° but this was not marked.

The practical importance of reviving phenol-treated bacteria has been stressed by Bennett (1959), and it would obviously be of value to test recovery media of this type on bacilli after treatment of spores with various phenolic compounds. The resistance to phenolic compounds of spores prepared by other methods, e.g. 7-day or 14-day old spores instead of 2-day old spores as used here, with or without pre-heating, could also be tested.

Pharmaceutics Laboratories,
Welsh School of Pharmacy,
Welsh College of Advanced Technology,
Cardiff.

MURIEL LOOSEMORE
A. D. RUSSELL

May 16, 1963

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Salicylate and Glutamate Metabolism

SIR,—Salicylate has been found to interfere with several important pathways involved in the metabolism of glutamate by animal tissues. The drug inhibits the synthesis of glutamine (Messer, 1958), glutamate-pyruvate transaminase activity (Steggle, Huggins and Smith, 1961), the enzymes responsible for the dehydrogenation and decarboxylation of the amino-acid (Gould, Huggins and Smith, 1963) and the incorporation of radioactive glutamate into muscle protein (Manchester, Randle and Smith, 1958). We have found that salicylate affects a further pathway of glutamate metabolism, its conversion to proline in preparations of rat costal cartilage. The drug also inhibited the incorporation of radioactive glutamate and proline into the cartilage protein but cortisol did not share these actions of salicylate.

Rat costal cartilage was freed from adherent muscle and cut into pieces, each of approximately 25 mg. Six pieces were incubated aerobically for 24 hr. at 37° in 1 ml. of the incubation medium of Salmon and Daughaday (1958) containing 50 μg. of Crystamycin (Glaxo) plus 2·5 μc of either [^{14}C]-glutamate or [^{14}C]-proline, in the presence or the absence of either 10 mM salicylate or 100 μg./ml. of cortisol. At the end of the incubation period the segments of cartilage were removed, washed for 10 sec. with fresh incubation medium, immersed in 0·5 ml. of boiling per cent (v/v) ethanol and allowed to extract for 24 hr. at room temperature. This process was repeated until no further radioactivity was extracted and all the aqueous ethanolic extracts were combined. The pieces of cartilage were then added to 2 ml. of 6N hydrochloric acid and heated in sealed glass tubes at 100° for 24 hr.; the hydrolysate was evaporated to dryness and the residue dissolved in 1 ml. of water. The radioactive substances present in the supernatants from the original incubation mixtures, in the aqueous ethanolic extracts of the cartilage and in the hydrolysates were separated by two dimensional chromatography and visualised by radioautography (Smith and Moses 1960). Radioactive glutamate and proline were presumptively identified by their chromatographic positions and this was confirmed by eluting the spots and separating the amino-acids by high-voltage paper electrophoresis, in the presence of authentic non-radioactive material, according to the directions of Atfield and Morris (1961). The ^{14}C in each spot was measured directly on the chromatograms by means of a Scott-type Geiger-Muller tube (Fuller, 1956).

Most of the carbon-14 from the labelled amino-acids which was incorporated into the soluble intermediates, appeared in substances such as malate, fumarate and aspartate. However, radioactive proline was detected in the supernatant, in the aqueous alcoholic extract and in the hydrolysate of the rat costal cartilage incubated with the ^{14}C-labelled glutamate. Only approximately 0·5 per cent of the glutamate carbons which had been metabolised by the tissue, appeared as proline and this conversion is therefore not a quantitatively important pathway for the metabolism of glutamate. The finding that glutamate carbons are incorporated into proline in rat costal cartilage is of interest since the overall conversion of glutamate does not seem to have been established *in vitro* with animal tissue although the intermediate reactions have been defined in microorganisms (Vogel. 1955) and nutritional and isotopic carbon studies (Sallach, Koeppe and Rose, 1951) suggest that glutamate is the precursor of proline in the intact rat.

The presence of cortisol did not reduce the extent of the conversion of the labelled glutamate to labelled proline but salicylate caused an approximately 50 per cent inhibition of the transfer of carbon-14 from the glutamate to proline. Strecker (1960) has suggested that the interconversion of glutamate and proline

in animal tissues may involve dehydrogenase enzymes requiring pyridine nucleotides as coenzymes. It has been shown (Bryant, Smith and Hines, 1963) that salicylate inhibits malate and isocitrate dehydrogenase activities by competing with either nicotinamide adenine dinucleotide or its phosphate and the drug may interfere with the biosynthesis of proline from glutamate by a similar mechanism. Trace amounts of radioactive glutamate were detected in the experiments in which the cartilage was incubated with the labelled proline; cortisol had no effect but in the presence of salicylate no radioactive glutamate was found.

TABLE I

EFFECTS OF SALICYLATE AND CORTISOL ON THE *IN VITRO* INCORPORATION OF RADIOACTIVE GLUTAMATE AND PROLINE INTO THE PROTEIN OF RAT COSTAL CARTILAGE

Labelled substrate	Amount of radioactive substrate (expressed as counts per min. × 10^{-3}) incorporated into the protein of 1 g. wet wt. of cartilage		
	Control	Salicylate	Cortisol
[^{14}C]-glutamate	177	89	192
[^{14}C]-proline	240	66	220

The results in Table I show that salicylate, but not cortisol, inhibited the incorporation of both the radioactive glutamate and the radioactive proline into the cartilage protein. Similar findings have been reported with isolated rat diaphragm in which salicylate reduced the incorporation of radioactivity from isotopically labelled amino-acids into the proteins (Manchester and others, 1958) whereas cortisol had no effect under similar experimental conditions (Manchester and Young, 1959).

We wish to thank Mr. D. Haylett for expert technical assistance and the Empire Rheumatism Council for a grant towards the cost of the work.

Empire Rheumatism Council Research Unit,
King's College Hospital Medical School,
Denmark Hill, London, S.E.5.

A. BELLAMY
A. K. HUGGINS
M. J. H. SMITH

June 12, 1963

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Sympathomimetic Activity of Guanethidine

SIR,—A report by Abercrombie and Davies (1963) indicated that guanethidine possesses direct sympathomimetic activity in addition to its catecholamine-depleting activity. Abboud and Eckstein (1962) reported that the vasodilator activity of guanethidine may be due to stimulation of β-adrenergic receptors. However, these investigators were not able to show significant changes in heart rate after guanethidine in animals treated with reserpine. In order to explore more fully this proposed sympathomimetic activity of guanethidine, the following experiments were made.

Mongrel dogs of either sex previously treated with reserpine were anaesthetised with 15 mg./kg. of pentobarbitone sodium and prepared for recording the following parameters. Femoral arterial blood pressure was recorded from the cannulated left femoral artery via a Statham pressure transducer on a Grass polygraph. Right femoral arterial blood flow was measured by placement of a Medicon flo-probe (2 mm. I.D.) around the vessel and recorded on the polygraph via a Medicon model FM-6 electromagnetic flowmeter. Chronotropic and inotropic activity were measured by suturing a Walton-Brodie strain gauge arch to the right ventricle after thoracotomy at the fourth intercostal space. The chest was sutured closed and the animal allowed to resume respiration by over-inflation of the lungs and induction of negative intrathoracic pressure. A 20 to 30 min. period elapsed before the administration of drugs.

TABLE I

EFFECT OF GUANETHIDINE, 15 MG./KG., ON CARDIAC ACTIVITY IN DOGS GIVEN RESERPINE BEFORE AND AFTER PRONETHANOL (NETHALIDE)

Treatment	Per cent of control response																	
	0·5 min.			1·0 min.			2·0 min.			3·0 min.			5·0 min.			10·0 min.		
	C	I	P	C	I	P	C	I	P	C	I	P	C	I	P	C	I	P
None	+37	+62	−28	+50	+30	−26	−54	+59	−24	+52	+59	−17	+46	+11	0	+23	−28	−17
Pronethanol 5 mg./kg., i.v.	+3	−14	−47	+1	−17	−22	+3	−20	+15	−5	−20	+34	−5	−17	+94	0	−11	+92

C, chronotropic activity; I, inotropic activity; P, peripheral blood flow calculated as peripheral resistance units; minus value indicates vasodilatation.

In a series of animals pre-treated with reserpine, guanethidine sulphate (15 mg./kg.) produced a biphasic blood pressure response. There was an initial and transient depressor response, of approximately 40 mm. Hg, lasting from 1 to 3 min. followed by a more sustained pressor response, of approximately 80 mm. Hg, lasting as long as 45 min. Accompanying the depressor response there were marked increases in both the chronotropic and inotropic activity of the myocardium, and a vasodilatation as seen in Table I. These effects decreased as the blood pressure reverted toward a pressor response.

To assess the possibility of β-receptor activity, pronethanol (5 mg./kg.), a specific β-receptor blocking agent, was injected intravenously before giving guanethidine. The effects on heart rate, ventricular force, and femoral blood flow are summarised in Table I. There was no change in the depressor response to guanethidine, however, the pressor response was markedly reduced. In addition, the chronotropic response was blocked and a negative inotropic response prevailed.

From these observations, it appears that guanethidine, in addition to its catecholamine-depleting activity, does possess some direct sympathomimetic action. However, the β-receptor mediated responses of heart rate and myocardial force of contraction appear to be dissociated from the peripheral vascular effects of this agent.

Department of Pharmacology,
School of Pharmacy,
University of Pittsburgh,
Pittsburgh 13, Pennsylvania, U.S.A.

EUGENE E. VOGIN
HAROLD SMOOKLER

May 22, 1963

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Cardiotonic Activity Amongst Polyene Antifungal Antibiotics

SIR,—The cardiotonic activity of hamycin and trichomycin, two polyene antifungal antibiotics, has been already reported (Arora, 1962, Arora and Sinha, 1963, Ozaki, Kataoka, Maesawa, Tshima and Kubo, 1954). Nystatin and lagosin, two more polyene antifungal antibiotics, revealed the presence of a similar activity.

Ten experiments were made with nystatin and lagosin on the perfused frog heart in which failure was induced by raising the venous pressure in steps of 1 cm. (Burn, 1952). Cardiac outflow was simultaneously recorded through a cannula in the aorta. Nystatin was perfused in a concentration of 2×10^{-5} g./ml. and lagosin in a concentration of 6×10^{-6} g./ml. With either drug, perfusion was followed by a marked increase in the amplitude of contraction as well as an increase in the cardiac outflow. This was followed by an increase in the diastolic tone and a decrease in the cardiac outflow, terminating in systolic arrest of the ventricles in 20 to 30 min. Effects were comparable with a concentration of 2×10^{-5} g./ml. of ouabain although the systolic contracture was not as complete as with nystatin or lagosin. Control experiments with propylene glycol for nystatin and ethanol for lagosin failed to produce the effects seen with these drugs.

Nystatin was kindly supplied by M/S. Squibb Institute for Medical Research, New Jersey, U.S.A., and lagosin, by Dr. V. Thaller, Oxford University.

Department of Pharmacology,
Maulana Azad Medical College,
New Delhi, India.

H. R. K. ARORA
M. K. BAGACHI

May 28, 1963

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Experimental Nephritis in Histamine- or 5-Hydroxytryptamine-depleted Rats

SIR,—Masugi (1933) evolved an experimental method for producing nephritis, by injecting specific nephrotoxic serum. Nephritis produced in this way appeared closely akin to human glomerulonephritis, in its clinical course and pathologic lesions (Seegal and Bevans, 1957). Sometimes symptoms resembling anaphylactic shock have also been produced (Baxter and Goodman, 1956). Preferential depletion of histamine and of 5-hydroxytryptamine (5-HT) from the rat, according to the method of Parratt and West (1957) did not affect the production of fatal anaphylactic shock (Sanyal and West, 1958), though contrary viewpoints regarding the role of histamine in anaphylaxis in this species have been reported (Mota, 1957). We have therefore been interested to discover whether the production of experimental nephritis could be influenced by previous depletion of histamine or 5-HT.

Kidneys from rats were perfused in saline to remove all traces of blood before being homogenised with saline to give about 1 g./ml. After the injection i.p. on 6–12 occasions at intervals of 1–2 days in rabbits, an antibody titre of serum was produced which was not less than 1 in 32, but usually between 1 in 64 to 1 in 128, as determined by a complement fixation test. Intravenous injection of 1 ml. of such sera per 100 g. of weight per rat was sufficient to produce histologically detectable changes.

The changes which were present in more than three-quarters of the animals, and appeared 24 hr. after treatment, consisted of swelling of epithelium of collecting tubules, distal and proximal convoluted tubules, almost blocking the lumen; dilatation of glomerular capillaries and oedema of interstital tissues. At 48 hr., the lesions were more marked, proliferation of glomerular tufts, adhesions between tufts and capsules were also present. In some animals epithelial crescents were seen.

Groups of 10–12 rats of either sex weighing about 100 g. obtained from local dealers were used for the main study. Animals were fed with "Anidiet" the composition of which has been previously described (Dhar and Sanyal, 1962). Water was allowed *ad lib.* One group of animals served as control, while one group received injections of polymyxin B to deplete tissue histamine, a third group received injections of reserpine to deplete 5-HT (Parratt and West, 1957).

Each of the animals then received an intravenous injection of the same batch of nephrotoxic serum. In the control group approximately 60 per cent of animals developed changes of nephritis; in the rest lesions, though present, were minimal.

In animals depleted of histamine by polymyxin injections, and then given nephrotoxic serum, kidney changes were insignificant in almost all animals.

Results obtained in animals depleted of 5-HT by injections of reserpine, were quite severe and of two types. In about half the animals, areas of coagulative necrosis were present. The lining epithelial cells of the convoluted tubules showed granular acidophilic cytoplasm with pyknotic or no nucleus. In other animals, granular material was seen in the glomerular spaces; those animals which showed considerable exudation had shrunken glomerular tufts: all stages of distintegration of glomerular tufts could be demonstrated.

Repeated injections of histamine, 5-HT or polymyxin did not produce significant histological changes in kidneys of rats.

Injections of reserpine in several species (rat, dog, guinea-pig, rabbit and mouse) failed to produce histological renal lesions. This is of significance,

since reserpine given before the nephrotoxic serum produced aggravated lesions.

This work was aided by a grant from the Indian Council of Medical Research.

Maulana Azad Medical College,
New Delhi, India.
May 20, 1963

RIKTA DAS GUPTA
H. L. DHAR
D. N. GUPTA
R. K. SANYAL

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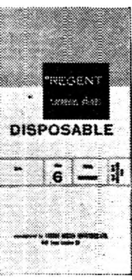

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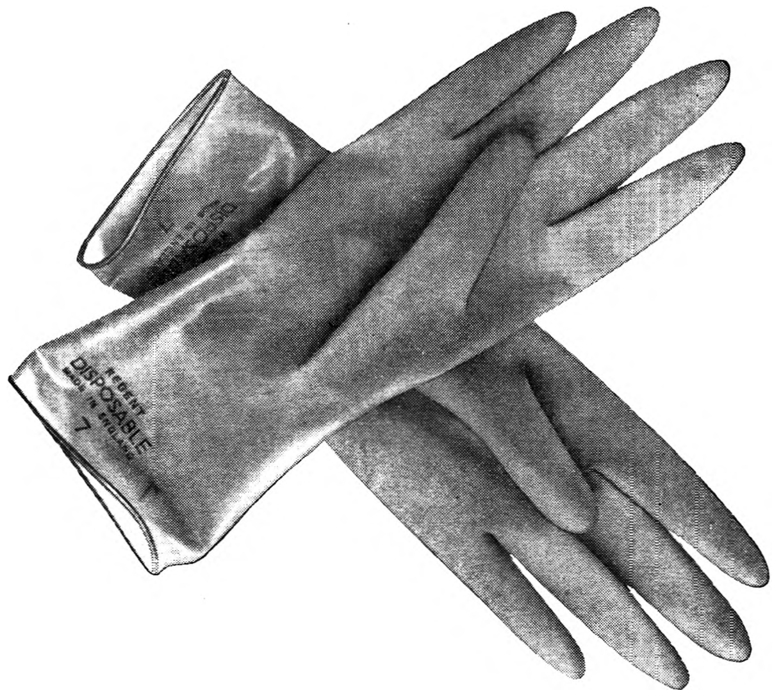

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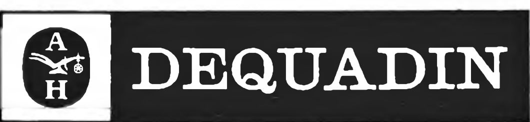

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