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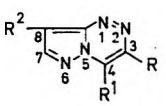
Science Papers

Pyrazolotriazines: a new class of tumour-inhibitory agents

R. W. BALDWIN, M. W. PARTRIDGE AND M. F. G. STEVENS*

A series of pyrazolo-*as*-triazines have been screened for tumour-inhibitory activity against sarcoma S 180 in mice and a methylcholanthrene-induced tumour in rats. The antitumour activities of ethyl 4-aminopyrazolo[3,2-*c*]-*as*-triazine-3-carboxylate (Ig), 6-acetyl- and 6-iodoacetyl-3-methyl-4-methylenepyrazolo[3,2-*c*]-*as*-triazine (IIa and IIb respectively) against sarcoma S 180 exceed the inhibitory activity of 6-mercaptopurine against the same tumour.

INVESTIGATIONS of structural analogues of naturally occurring purines have provided a fruitful source of compounds showing inhibitory activity against a range of experimental animal neoplasms. Thus the antitumour activities and structure-activity relationships of derivatives of purine, pyrazolo[3,4-*d*]pyrimidine and *v*-triazolo[*d*]pyrimidine have received widespread interest in the search for cancer chemotherapeutic agents (Robins, 1964). Pyrazolo[3,2-*c*]-*as*-triazine (I; R = R^1 = R^2 = H) is isosteric with purine and the synthesis of compounds based on this ring-system has already been described (Partridge & Stevens, 1966; Bedford, Partridge & Stevens, 1966). This communication reports on studies of the tumour-inhibitory properties of the compounds listed in the Tables.



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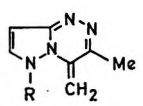

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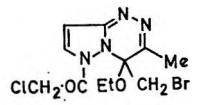

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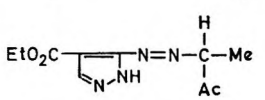

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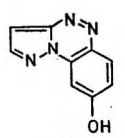

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Experimental

METHODS

The procedure for testing compounds against mouse sarcoma S 180 was based on the protocol of the United States Cancer Chemotherapy Service Centre (1962). Tumour (7 to 10 days old) was taken under aseptic

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conditions from donor mice and fragments (2–4 mm in average diameter) were implanted subcutaneously using a 9 gauge trocar into stock albino mice (Schofield; 25–30 g weight).

Soluble compounds were administered in physiological saline. Insoluble compounds were suspended by homogenisation in 0·5% carboxymethyl cellulose in physiological saline.

Treatment was begun 24 hr after tumour implantation and compounds were administered daily by intraperitoneal injection for 9 days (days 1–9). Mice were weighed and killed (day 10) and the tumours excised and weighed. The ratio of the mean weights of tumours in treated mice to that in controls (T/C × 100%) was recorded together with body weight changes.

A limited number of tests were also made with a methylcholanthrene-induced rat sarcoma (Baldwin, 1955). This tumour was carried by subcutaneous implantation in female rats of an inbred Wistar strain, and was used between the 65th and 103rd transfer generations. In these tests, tumour fragments (5–10 mm in average diameter) were implanted subcutaneously using a 15 gauge trocar, and compounds were administered daily by intraperitoneal injection for 17 days (days 1–17). Rats were then killed 24 hr after the last treatment and inhibition determined from the average weights of tumour in test and control animals.

Results and discussion

Derivatives of pyrazolo[3,2-*c*]-*as*-triazine (I) with substituents in the 3-, 4- and 6- positions differed in their abilities to inhibit the growth of sarcoma S 180 (Table 1). The monomethyltriazine (Ia) was inactive, whereas its dimethyl analogue (Ib) showed activity compared to the positive controls; these were treated with 6-mercaptopurine and *N*-methylformamide. Introduction of a bromine atom into the pyrazole ring abolished activity (Ic); similar deactivation resulted from the replacement of methyl by phenyl (Id). The dyschemotherapeutic effect of a substituted 3-carbonyl (Ie and If) was offset by a 4-amino-group (Ig and Ih), the ester (Ig) being somewhat more active than the corresponding acid (Ih). The chemical reactivity of the 4-amino-group of (Ig) was exploited to synthesise a compound combining the structural features of a purine analogue and a nitrogen mustard (Partridge & Stevens, 1966); the resulting 4-di-β-chloroethylaminopyrazolotriazine (Ii) was however inactive. Sarcoma S 180 is known to be resistant to biological alkylating agents. Moreover, the biological activities of nitrogen mustards are related to the chemical reactivity of their halogen atoms (Ross, 1962). The ethoxycarbonyl group in (Ii) adjacent to the di-β-chloroethylamino-group would deactivate the chlorine atoms to nucleophilic attack.

The 6-acetyltriazine (IIa) was tumour inhibitory. The chemical reactivity of 6-acyl derivatives towards nucleophilic reagents to yield 3,4-dimethylpyrazolo[3,2-*c*]-*as*-triazine (Ib) (Partridge & Stevens, 1966) implied the possibility that the 6-acetyl derivative might exert its activity after *in vivo* metabolism to (Ib). The feasibility of exploiting such metabolism by the incorporation of a biologically active side-chain at the

6-position of (Ib) in order to potentiate cytotoxicity was investigated. Two derivatives, 6-iodoacetyl- and 6-fluoroacetyl-3-methyl-4-methylene-pyrazolo[3,2-*c*]-*as*-triazine (IIb and IIc respectively) were significantly more toxic to the mouse than the corresponding 6-acetyltriazine (IIa); this suggested that *in vivo* (IIb and IIc) were degraded to the dimethyl-triazine (Ib) and iodo- and fluoroacetic acids respectively. The iodo-acetyltriazine (IIb) was a more potent inhibitor of sarcoma S 180 than

TABLE 1. INHIBITION OF SARCOMA S 180 BY PYRAZOLOTRIAZINES

Compound	Substituents R	R¹	R²	Dosage (mg/kg/day)	Survivors	Average body-weight change, test/control (g)	Mean tumour weights, test/control (g)	Tumour inhibition, mean tumour weights, test/control (%)
6-Mercapto-purine				30	5/6	+1·4/+5·9	0·66/0·95	69
N-Methyl-formamide				200	6/6	+1·5/+8·2	0·62/1·42	44
				200	6/6	+0·2/+7·0	0·38/1·33	29
Ia	H	Me	H	200	6/6	+2·8/+6·2	1·1 /0·92	120
Ib	Me	Me	H	200	6/6	+2·5/+3·5	0·68/1·03	66
				200	6/6	+1·0/+6·2	0·76/0·92	83
Ic	Me	Me	Br	50	6/6	+2·5/+3·2	0·95/0·60	158
Id	Ph	Ph	H	100	6/6	+5·8/+6·2	3·21/2·07	155
Ie	Ac	Me	H	50	6/6	+1·7/+1·0	0·70/0·64	109
If	CO_2Et	Me	H	50	5/6	+2·4/+4·8	0·99/1·01	98
Ig	CO_2Et	NH_2	H	25	5/6	+2·8/+4·2	0·72/1·39	52
				25	5/6	+0·8/+4·8	0·65/1·48	44
Ih	CO_2H	NH_2	H	10	6/6	+3·2/+6·2	1·47/2·07	72
Ii	CO_2Et	$N([CH_2]_2Cl)_2$	H	25	6/6	+2·5/+2·7	0·40/0·32	125
IIa	Ac			200	6/6	+1·3/+0·3	0·17/0·46	37
				200	6/6	+6·0/+5·8	0·63/1·01	62
				200	6/6	+2·5/+1·0	0·43/0·66	68
IIb	ICH_2CO			5	6/6	+0·5/+2·0	0·75/1·30	58
				7·5	6/6	+0·3/+6·5	0·48/0·87	55
				10	5/6	+0·4/+3·7	0·33/0·93	35
IIc	FCH_2CO			10	6/6	−1·0/+2·5	0·39/0·40	98
IId	$ClCH_2CO$			50	5/6	+1·2/+3·3	0·88/0·93	95
				100	6/6	−5·0/+0·0	0·45/0·89	50
IIe	Cl_2CHCO			200	6/6	+0·3/+4·2	1·00/1·39	72
IIf	$PhCO$			100	6/6	+2·7/+4·7	1·07/0·74	144
IIg	$PhSO_2$			200	6/6	+0·0/+1·2	1·25/1·32	94
IIh	*p*-$AcNHC_6H_4SO_2$			100	6/6	+2·5/+1·3	0·58/0·83	70
IIi	phthalimidoacetyl			100	6/6	+0·8/+2·0	0·96/1·30	74
IIj	4-NC_5H_4CO			100	6/6	+3·7/+4·7	0·41/0·32	128
III				100	5/6	+0·0/+6·5	0·63/0·89	72
IV				100	6/6	+1·8/+1·3	0·52/0·83	63
				100	6/6	+1·2/+2·0	0·92/1·30	71
V				100	6/6	+5·2/+4·8	0·64/0·63	102

6-mercaptopurine at the three dose levels tested, but surprisingly the fluoroacetyl derivative (IIc) was inactive. The monochloroacetyltriazine (IId) and its dichloro analogue (IIe) were tumour-inhibitory: however the inhibitory dose of the monochloro derivative produced excessive body-weight loss in the test animals. Of the remaining 6-substituted compounds (IIf-j, and III), three triazines (IIh, IIi and III) showed inhibitory properties; the activity of compound (III) can possibly be attributed to its metabolic degradation to 3,4-dimethylpyrazolo[3,2-*c*]-*as*-triazine (Ib), since compound (III) is labile to acid and alkali (Bedford, Partridge & Stevens, 1966). The pyrazole-azobutanone (IV) displayed similar activity to 6-mercaptopurine but the pyrazolobenzotriazine (V) was inactive.

Both the dimethyltriazine (Ib) and its acetyl derivative (IIa) which inhibit sarcoma S 180, inhibit a methylcholanthrene-induced tumour in rats (Table 2). The inactivity of the ester (If) against sarcoma S 180 was paralleled by its inactivity against the methylcholanthrene tumour, but the amine (Ig) was less active against this tumour than against S 180.

TABLE 2. INHIBITION OF A METHYLCHOLANTHRENE-INDUCED TUMOUR BY PYRAZOLOTRIAZINES

Compound	Substituents R	R¹	R²	Dosage (mg/kg/day)	Survivors	Average body-weight change, test/control (g)	Mean tumour weights, test/ control (g)	Tumour inhibition, mean tumour weights, test/ control (%)
Ib*	Me	Me	H	100	4/4	+14·3/+25·8	5·57/14·77	38
				200	5/6	+5·4/+12·5	1·89/ 5·12	37
If	CO_2Et	Me	H	50	6/6	+14·0/+10·0	6·93/ 4·86	143
Ig	CO_2Et	NH_2	H	25	6/6	+2·3/+11·3	3·96/ 4·86	82
IIa†	Ac			200	6/6	−2·9/+0·8	1·83/ 7·89	24
				200	6/6	−5·0/+10·0	2·96/ 4·86	61

* This compound (dose, 160 mg/kg/day) also inhibited the Walker carcinoma carried in male Wistar rats (T/C = 66).
† The acetyltriazine (dose, 200 mg/kg/day) inhibited growth of the Walker carcinoma (T/C = 64)

Although too few pyrazolo[3,2-*c*]-*as*-triazines have been studied to draw any significant structure-activity conclusions, the preliminary screening reported in this paper indicates that several derivatives show anti-tumour properties.

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SHORT COMMUNICATION

The relationship between thin-layer chromatographic behaviour and the stereochemistry of some heteroyohimbine alkaloids

J. D. PHILLIPSON AND E. J. SHELLARD

A NUMBER of thin-layer chromatography systems have been used to distinguish and identify some indole and oxindole alkaloids isolated from various species of the genus *Mitragyna* (Shellard & Phillipson, 1964). It has been suggested moreover that a relationship exists between the stereochemistry of these alkaloids and their behaviour on thin-layer chromatograms (Phillipson & Shellard, 1966). Two new heteroyohimbine alkaloids, mitrajavine and hirsutine, have recently been isolated from *Mitragyna* species (Shellard, Beckett, Tantivatana, Phillipson & Lee, 1966) and their behaviour on thin-layers has been compared with some related alkaloids of known stereochemistry. Based on this, certain suggestions have been made about the stereochemistry of these two new alkaloids.

EXPERIMENTAL

Details of plate preparation, development of the chromatograms and detection of the alkaloids have already been published (Phillipson & Shellard, 1966). The following systems were used: 1, silica gel, benzene–ethyl acetate (7:2); 2, alumina, chloroform–benzene (1:1); 3, alumina, chloroform–benzene–diethylamine (1:1:0·001); 4, alumina, benzene–ethyl acetate (7:2); 5, alumina, ether; 6, silica gel, ether; 7, silica gel, chloroform–acetone (5:4); 8, alumina, cyclohexane–chloroform (3:7); 9, alumina, cyclohexane–chloroform–diethylamine (3:7:0·005); 10, silica gel, benzene–ethyl acetate–diethylamine (7:2:1).

RESULTS

The results of the separations obtained are illustrated in Fig. 1A and B. The Rf values were determined by averaging the results from six separate determinations.

DISCUSSION

It has been proposed that all naturally occurring heteroyohimbine alkaloids of the corynantheidine type (I) and of the ajmalicine type (II) have C(15)-Hα as a common stereochemical factor (Wenkert & Bringi, 1959). Individual alkaloids of each type may differ in the following particulars:

(*a*) Substituents in positions 9, 10, 11, 12 (I, II).

(*b*) Different configurations at C(3) and C(20) giving four possible isomers classified thus: allo, C(3)-Hα; C(20)-Hα: epiallo, C(3)-Hβ;

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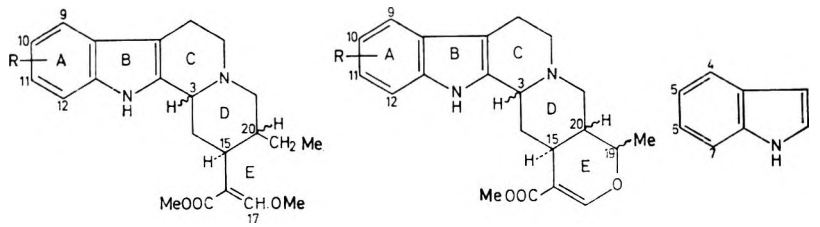

I II III

C(20)-Hα: normal, C(3)-Hα; C(20)-Hβ: pseudo, C(3)-Hβ; C(20)-Hβ (Saxton, 1956, 1960).

(*c*) C(17)-H *cis* or *trans* to the carbomethoxy group in E seco alkaloids (I).

(*d*) C(19) - Me α or β in closed E ring alkaloids (II).

Six heteroyohimbine alkaloids (I) of known stereochemistry have been examined by the ten thin-layer systems given above. It has been proposed

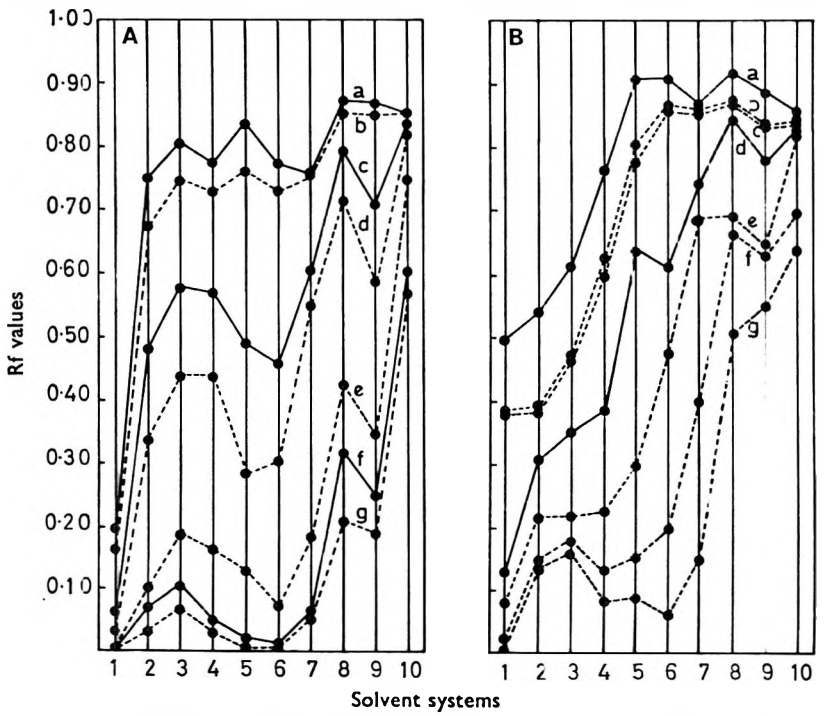

FIG. 1A. Rf values of (*a*) corynantheidine (I, R = H, allo), (*b*) mitragynine (I, R = 9 — OMe, allo), (*c*) dihydrocorynantheine (I, R = H, normal), (*d*) speciogynine (I R = 9 — OMe, normal), (*e*) speciociliatine (I, R = 9 — OMe, epiallo), (*f*) hirsutine (I, R = H), (*g*) mitraciliatine (I, R = 9 — OMe, pseudo).

FIG. 1B. Rf values of (*a*) tetrahydroalstonine (II, R = H, allo), (*b*) aricine (II, R = 10 — OMe, allo), (*c*) reserpinine (II, R = 11 — OMe, allo), (*d*) ajmalicine (II, R = H, normal), (*e*) tetraphylline (II, R = 11 — OMe, normal), (*f*) isoreserpinine (II, R = 11 — OMe, epiallo), (*g*) mitrajavine (II, R = 9 — OMe).

that these alkaloids have the C(17)-H *cis* to the carbomethoxy group as a common stereochemical factor (Weisbach, Kirkpatrick, Williams, Anderson, Yim & Douglas, 1965; Phillipson, 1965; Lee, Trager & Beckett, personal communication). The alkaloids differ from each other as follows: corynantheidine (I, R = H), allo (van Tamelen, Aldrich & Katz, 1956); mitragynine (I, R = 9-OMe), allo (Joshi, Raymond-Hamet & Taylor, 1963; Zacharias, Rosenstein & Jeffrey, 1965); dihydrocorynantheine (I, R = H), normal (Wenkert & Bringi, 1959); speciogynine (I, R = 9-OMe), normal; speciociliatine (I, R = 9-OMe), epiallo; mitraciliatine (I, R = 9-OMe), pseudo (Lee, Trager & Beckett, personal communication).

Fig. 1 shows that when the 9-methoxy substituted alkaloids are arranged in order of decreasing Rf values, the sequence is allo, normal, epiallo and pseudo. The allo and normal alkaloids (I, R = H) have higher Rf values than the two 9-methoxy alkaloids with the corresponding stereochemistry. Hirsutine has been shown to be an isomer of the corynantheidine type (I, R = H) (Shellard & others, 1966). If it is assumed that hirsutine has the same stereochemistry across the double bond as the other six alkaloids, i.e. C(17)-H *cis* to the carbomethoxy group, then hirsutine can differ from corynantheidine (allo) or dihydrocorynantheine (normal) by being an epiallo or pseudo compound. Fig. 1 shows that in the ten thin-layer systems used, hirsutine has slightly higher Rf values than mitraciliatine but lower Rf values than speciociliatine. These observations suggest that hirsutine is stereochemically similar to mitraciliatine in having the pseudo configuration.

Six heteroyohimbine alkaloids of the ajmalicine type (II) and of known stereochemistry have been examined by the same ten thin-layer systems. The alkaloids differ from each other as follows: tetrahydroalstonine (II, R = H), allo; aricine (II, R = 10-OMe), allo; reserpinine (II, R = 11-OMe), allo; ajmalicine (II, R = H), normal; tetraphylline (II, R = 11-OMe), normal; isoreserpinine (II, R = 11-OMe), epiallo (Wenkert, Wickberg & Leicht, 1961; Shamma & Moss 1961, 1962). C(19)-Meα is a common stereochemical factor in these alkaloids.

Fig. 1B shows that when the 11-methoxy substituted alkaloids are arranged in order of decreasing Rf values then the sequence of allo, normal and epiallo is the same as with the E seco alkaloids. It is probable therefore, that the behaviour of the pseudo alkaloid in this series would be similar in having the lowest Rf values. The allo and normal alkaloids (II, R = H) have higher Rf values than the two 11-methoxy alkaloids (II, R = 11-OMe) with the corresponding stereochemistry. When indole (III) and some methoxy indoles were examined by thin-layer systems 1–6, the sequence in order of decreasing Rf value was indole, 4-, 5- and 6-methoxyindole. If the thin-layer behaviour of these simple indoles can be related to the heteroyohimbine alkaloids then the expected sequence in order of decreasing Rf value would be 9-, 10- and 11-methoxy The case of aricine (II, R = 10-OMe, allo) which has Rf values slightly higher than those of reserpinine (H, R = 11-OMe, allo), agrees with this suggestion.

Mitrajavine has been shown to be of the ajmalicine type (II, R = 9-OMe) (Shellard & others, 1966). It would be expected that on these ten thin-layer systems, the Rf values of mitrajavine would be higher than those of the 11-methoxy substituted alkaloid with an identical stereochemistry. Fig. 1B shows that mitrajavine has lower Rf values than the 11-methoxy substituted alkaloids of the allo, normal and epiallo configurations, thus *if it be assumed that mitrajavine has a C*(19)-*Me α-configuration*, then its behaviour on these ten thin-layer systems suggests that it is a pseudo compound.

These results tentatively suggest that in heteroyohimbine alkaloids there appears to be a relationship between alkaloidal structure and thin-layer chromatographic behaviour, as follows:

1. Methoxy substituents lower Rf values.
2. Rf values of 10-substituted alkaloids of allo configuration and with a closed E ring (II), are slightly higher than the corresponding 11-substituted alkaloid.
3. The E seco alkaloids (I) can be arranged in order of decreasing Rf values as allo, normal, epiallo and pseudo.
4. The closed E ring alkaloids (II) can be arranged in order of decreasing Rf values as allo, normal and epiallo.

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SHORT COMMUNICATION

Determination of the homologue composition of some alkyltrimethyl quaternary ammonium antibacterial agents by gas chromatography

H. H. LAYCOCK AND B. A. MULLEY

RECENT work has shown that the micellar properties of quaternary ammonium surface-active agents may be linked with their antibacterial action (Weiner, Hart & Zografi, 1965). Micelle formation and antibacterial activity both depend markedly on the chain length of the hydrocarbon groups attached to the quaternary nitrogen, and other biological properties may also depend on this aspect of their structure (Hart & Nissim, 1966). Commercially produced materials are mixtures of homologues and a method is described below for measuring the homologue composition.

Previous work (Metcalfe, 1963) has described the gas chromatography of long chain quaternary ammonium compounds on an alkaline column. Under the published conditions a mixture of the corresponding tertiary amines was produced, the analysis of which provided a measure of the homologue composition of the original material. We were unable to reproduce these experiments but the following method proved successful. Two types of column were used and results seem to be essentially the same for both. Column A consisted of 10% silicone elastomer (S.E. 30) on kieselguhr, whilst column B consisted of a 5 ft column packed for its initial 2 ft with 20% potassium hydroxide on kieselguhr followed by 10% potassium hydroxide + 10% Apiezon L on the same support for the remainder of its length. The long chain quaternary ammonium compound in the form of its hydroxide was used in the analysis. Under the conditions described in the experimental section the normal β-elimination took place to yield an olefin, accompanied by the alternative mode of decomposition, namely loss of methanol, to give the corresponding tertiary amine. When a pure sample of tetradecyltrimethylammonium bromide was analysed in this way two peaks resulted, one

TABLE 1. HOMOLOGUE COMPOSITION OF SOME ALKYLTRIMETHYL QUATERNARY AMMONIUM COMPOUNDS

Compound	Approximate composition %				
	$C_{(10)}$	$C_{(12)}$	$C_{(14)}$	$C_{(16)}$	$C_{(18)}$
Sample A (cetrimide)	—	24	65	9	2
" B "	—	27	62	11	—
" C "	—	21	69	10	—
Morpan T	1	9	86	2	2
" CHA	1	6	10	69	14
" O	—	7	4	3	86

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from 1-dimethylaminotetradecane and the other from tetradec-1-ene. The peaks were identified by using standards of the authentic substances; decomposition was shown to be quantitative. The result of analysing cetrimide B.P. and some other samples of quaternary ammonium compounds in this way is shown in Table 1. A typical chromatogram (cetrimide sample) is shown in Fig. 1. The approximate percentage of each component present was expressed as a percentage of the total peak area (internal normalisation). Errors involved in applying this method are probably not serious since the detector response variation within a homologous series for a flame ionisation detector is small (Warrington, 1961) and the difference in response between olefin and amine was shown to be about 5% for equal weights of dimethylaminotetradecane and tetradec-1-ene.

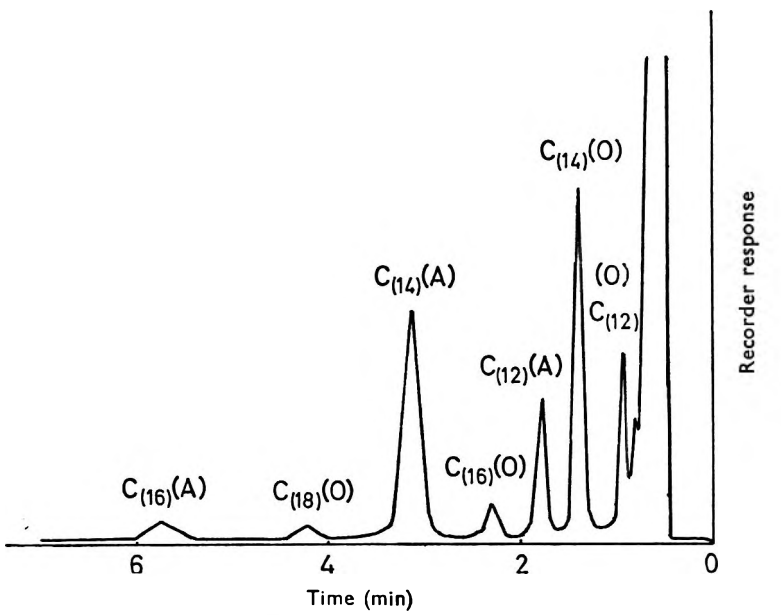

FIG. 1. Chromatogram of cetrimide, Sample A. (O) = olefin; (A) = tertiary amine.

The results for the cetrimide samples show that these products contain about 25% of $C_{(12)}$ compounds. The effect of this is to improve the solubility of the products although recent results (Weiner & others, 1965) suggest that it may also affect their antibacterial activity. Results for two of the Morpan samples in the Table agree reasonably well with the homologue composition of the alcohols used in the production of the compounds (see also the experimental section). The unsaturated components present in Morpan CHA were not detected under the conditions used.

MATERIALS

Tetradecyltrimethylammonium bromide was kindly supplied by Mr. J. E. Adderson (Adderson & Taylor, 1964). *Cetrimide B.P. samples.* Sample A was obtained from Glovers (Chemicals) Ltd., samples B and C from Imperial Chemical Industries Ltd. *Morpans* were donated by Glovers (Chemicals) Ltd. The percentage composition of the alcohols from which they were prepared was as follows: Morpan T, $C_{(14)}$ 95; Morpan CHA, $C_{(14)}$ 10, $C_{(16)}$ 70, $C_{(18)}$ 17, unsaturated material 3. *Tertiary amines and* 1*-alkenes* ($C_{(10)}$–$C_{(18)}$) were obtained from Kodak Ltd. The $C_{(14)}$ compound of each series was purified by preparative gas chromatography. Analysis of the other samples showed that several homologues were present and the retention times of the main peaks were determined. Their identities were checked by plotting the logarithm of the retention times against the number of carbon atoms in the chain when a linear plot was obtained.

APPARATUS AND METHODS

(i) A Pye "Series 104", model 4 flame ionisation isothermal chromatograph was used for the analysis of the pure sample of tetradecyltrimethylammonium bromide. A 4 mm diameter circular, stainless steel column, 5 ft long, was packed with acid-washed Celite (80/100 mesh) coated with 10% w/w S.E. 30. Column temperature, 240°; injection heater, 290°; carrier gas nitrogen, 20 ml/min.

0·003 ml of a test solution containing approximately 2% w/w tetradecyltrimethylammonium bromide and a reference solution containing 0·52% w/w tetradec-1-ene and 1-dimethylaminotetradecane respectively were used. In each case the solvent—methanol—contained 2% w/w potassium hydroxide.

(ii) A Pye Panchromatograph, with a flame ionisation detector was used for the cetrimide and Morpan samples. A 4 mm diameter glass column, 5 ft long, was packed for 3 ft with acid-washed Chromosorb W (60–80 mesh) coated with 10% w/w Apiezon L and 10% w/w potassium hydroxide, and for the initial 2 ft with Chromosorb W coated with 20% w/w potassium hydroxide. Column temperature, 240°; carrier gas argon at approximately 60 ml/min 0·005 ml of solutions each containing 4% w/w of the quaternary ammonium compound and approximately 1·2% w/w potassium hydroxide in methanol were injected.

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The determination of fluocinolone acetonide in formulated products

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A chromatographic procedure for the determination of fluocinolone acetonide is described. The method employs a hexane : dioxan : water partition system supported on Celite. The progress of the chromatogram is followed by measurement of the ultraviolet absorption of eluate fractions at 238 mμ. The application of the method to the determination of fluocinolone acetonide in cream, ointment and lotion formulations is described.

SYNTHETIC corticosteroids containing both a dihydroxyacetone side-chain and an α,β-unsaturated carbonyl function have been in use for many years for the treatment of inflammatory conditions. Published procedures (B.P. 1958; Mader & Buck, 1952; Umberger, 1955) for the determination of these substances in creams, lotions and ointments have depended almost exclusively on either the reducing properties of the former grouping or the chromophoric nature of the latter. The high activity of fluocinolone acetonide enables the use of preparations containing but a fraction of the steroid content of those formerly employed and difficulty in applying established corticosteroid assay techniques was encountered. This is due in part to complexity of formulation, and in certain instances to the presence of antibiotics.

Experimental

Preliminary experiments confirmed that techniques involving oxidation of the dihydroxy acetone side-chain by tetrazolium salts (Mader & Buck, 1952), ultraviolet light absorption or polarographic measurement (Kolthoff & Lingane, 1952) of the α,β-unsaturated carbonyl function could be applied to the parent compound whereas methods based on condensation with isonicotinic acid hydrazide (Umberger, 1955) failed to give stoichiometric results.

All the above methods suffered severe interference from excipients when applied to cream, lotion and ointment formulations, and classical solvent extraction methods failed to provide extracts sufficiently free from interfering material for any to be applied with success.

It was established, however, that fluocinolone acetonide was adsorbed from chloroform solution by both activated alumina and silica gel. Elution from the former was accomplished only by using the most polar solvents, and recovery of the steroid was never complete. The latter adsorbent was suitable only for extracts from the simplest formulation, as the degree of adsorption was markedly influenced by traces of extracted dispersing agent. A partition system similar to that used for triamcinolone (Smith, Foell, de Maio & Halwer, 1959) was therefore explored. An equilibrated mixture of hexane - dioxan - water (100 : 40 : 5) was used

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as a solvent; the lower layer incorporated with Celite formed the stationary phase, and the upper layer was used as developing solvent. The progress of the chromatogram was followed by measuring the absorbance at 238 mμ* of successive fractions of column eluate. The relationship between absorbance and volume of eluate for a purified sample of fluocinolone acetonide is illustrated in Fig. 1.

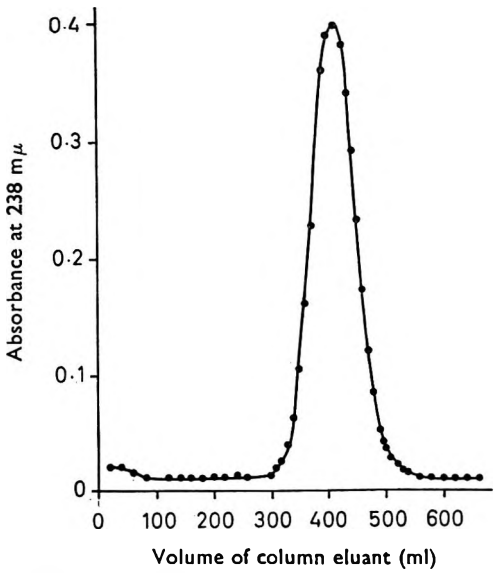

FIG. 1. Curve relating absorbance with volume of eluate for pure fluocinolone acetonide.

METHOD

Reagents: Prepared Celite. Stir Celite 545 (500 g) intermittently for 12 hr with concentrated hydrochloric acid (2 litres). Decant the bulk of the hydrochloric acid, and suspend the residue in about 1 litre of water. Filter through a Buchner funnel and wash with water until free from acid. Wash with methanol (500 ml) and finally with methanol-ethyl acetate (1 : 1) (1 litre). Dry in an oven at 100° and transfer to well stoppered jars. *Hexane.* Commercial samples of n-hexane, b.p. 67–69°, having an absorbance against air of 0·7 or less at 238 mμ do not normally require pretreatment. Poor samples can be rendered satisfactory by oleum washing followed by distillation.

Dioxan. Analar grade material has always been found satisfactory but certain other selected batches of less expensive material have proved an acceptable substitute.

* This represents the wavelength of maximum absorption of fluocinolone acetonide in eluent phase.

PREPARATION OF SOLVENT SYSTEM

Shake together n-hexane (1000 ml), dioxan (400 ml), water (50 ml) and allow to separate. The upper layer is the eluent phase, the lower layer the stationary phase.

PREPARATION OF SAMPLE

(1) *Cream and lotion* 0·025%. Transfer an accurately weighed quantity of sample (about 10 g to a 250 ml separating funnel with methanol (50 ml). Add cyclohexane (100 ml) and shake vigorously (3 min). Transfer the lower layer to a 500 ml separating funnel containing water (140 ml), add chloroform (100 ml) and shake vigorously (3 min). Filter the lower layer through a No. 1 Whatman filter paper and evaporate 50·0 ml of the filtrate to dryness on a steam bath in a current of air. Dissolve the residue in stationary phase (1 ml) and reserve for the chromatographic stage.

(2) *Ointment* 0·025%. Proceed as described above using methanol - water (5 : 1) (60 ml) in place of methanol (50 ml) in the primary extraction stage.

(3) *Cream, lotion and ointment* 0·01%. Take 12–14 g sample and proceed as described under appropriate section above, using a 75·0 ml aliquot of the final chloroform filtrate.

(4) *Cream, lotion and ointment* 0·025% *and* 0·01% *containing neomycin sulphate*. Carry out the preliminary partition between cyclohexane and aqueous methanol as described under the appropriate fluocinolone acetonide preparation. Transfer the lower (aqueous methanol) layer as completely as possible to a 500 ml separating funnel taking care to exclude any flocculent precipitate of neomycin sulphate present at the interface. Add methanol - water (4 : 1) (25 ml) to the residual cyclohexane layer and shake for 3 min. Combine the lower layers, add water (215 ml), chloroform (100 ml) and proceed as described under the appropriate preparation without neomycin sulphate.

PREPARATION OF STANDARD

Dissolve an accurately weighed quantity of pure fluocinolone acetonide (about 20 mg) in chloroform (100 ml.)

PREPARATION AND STANDARDISATION OF CHROMATOGRAPHIC COLUMN

Mix prepared Celite (15 g) with stationary phase (7·5 ml) and pack into a chromatographic column (80 cm in length, 2·2 cm internal diameter, fitted with a sinter plate and a bottom glass tap) in portions of about 3 g: pack down firmly with a tamper between each addition. Evaporate standard fluocinolone acetonide solution 5 ml, to a dryness in a current of air. Dissolve the residue in stationary phase (1·0 ml), add prepared Celite (2 g), mix thoroughly and pack onto the top of the stationary phase. Complete the transfer of any material remaining with the aid of 50 ml of eluent phase, ensuring the minimum disturbance of the column packing. Add eluent phase carefully to the column to a depth of about 50 cm and maintain this level throughout the chromatogram, adjust the

flow rate to 8–10 ml of eluent per min and collect 60 successive fractions of eluate. Measure the absorbance at 238 mμ of each fraction against eluent phase in the reference cell.

Chromatography of sample. Add prepared Celite (2 g) to the sample residue dissolved in stationary phase (1 ml) and pack on a fresh stationary phase. Complete the chromatogram in the manner previously described.

The fluocinolone acetonide content of the sample $= \frac{Ea \times Ws \times 100}{Es \times Wa \times V} \times 100\%$ where Ea and Es are the sums of absorbance values under the sample and standard peaks respectively, after correction for any base line blank values. Wa = weight of sample (mg); Ws = weight of standard fluocinolone acetonide (mg) applied to column; V = volume of chloroform extract (m) evaporated to dryness before application to column.

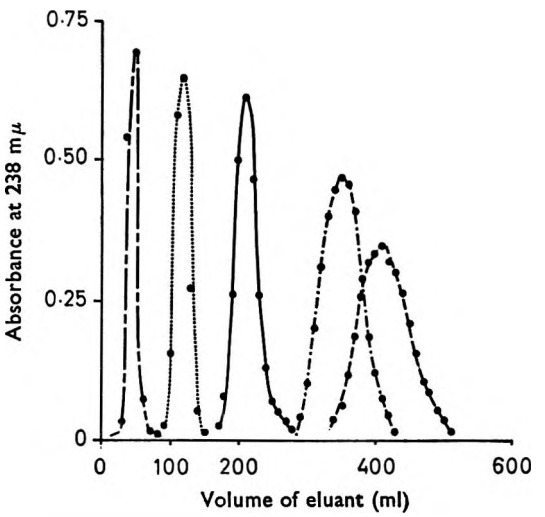

FIG. 2. Curves relating absorbance volume for fluocinolone acetonide and other currently available corticosteroids. — — — Betamethasone 17-valerate. · · · · Hydrocortisone acetate. ——— Prednisolone acetate. –·–·–·– Triamcinolone acetonide. — — — — Fluocinolone acetonide.

Results and discussion

Preservatives such as *p*-hydroxybenzoic and gallic acid esters or dispersing agents of the anionic and non-ionic variety do not interfere with the proposed assay for fluocinolone acetonide. Likewise the degradation products resulting from oxidation of the 11β-hydroxy group or rearrangement of the C(17) side-chain were without effect.

The method has been applied to samples of other anti-inflammatory corticosteroids including betamethasone 17-valerate, hydrocortisone, hydrocortisone acetate, prednisolone and prednisolone acetate and the plot of extinction at 238 mμ versus eluate volume is illustrated in Fig 2. With the exception of hydrocortisone and prednisone all the examples cited are

eluted in the first 600 ml and the degree of separation is such that confusion between individual members is unlikely. The retention volume of individual members can readily be adjusted to an optimum by variation of the dioxan to water ratio of the solvent system; this is particularly necessary for the two less polar members of the group. The system hexane-dioxan-methanol-water (100:30:5:5) gives a retention volume of 200 ml and has been used successfully for the analysis of betamethasone 17-valerate formulations after identical pretreatment to that described for the appropriate fluocinolone acetonide preparation.

TABLE 1. RESULTS OBTAINED BY THE PROPOSED METHOD ON LABORATORY PREPARED FLUOCINOLONE ACETONIDE FORMULATIONS

Sample		Fluocinolone acetonide % w/w			Sample		Fluocinolone acetonide % w/w			
		Added	Found				Added	Found		
Fluocinolone	1.	0·025	0·025	0·025	Fluocinolone	16.	0·C24	0·024	0·025	0·024
acetonide lotion	2.	0·024	0·024	0·024	acetonide cream	17.	0·C25	0·025	0·025	0·025
	3.	0·024	0·024	0·024	with neomycin	18.	0·C25	0·024		
	4.	0·010	0·010	0·009		19.	0·C25	0·025		
	5.	0·010	0·010	0·010		20.	0·C10	0·011		
Fluocinolone	6.	0·025	0·024	0·025		21	0·009	01·00		
acetonide lotion	7.	0·023	0·023			22.	0·009	0·009		
with neomycin	8.	0·025	0·025	0·025	Fluocinolone	23.	0·C25	0·024	0·024	
	9.	0·024	0·025		acetonide ointment	24.	0·C25	0·025	0·023	
	10.	0·024	0·024			25.	0·C25	0·025		
	11.	0·011	0·011			26.	0·C24	0·023		
	12.	0·010	0·009			27.	0·C10	0·010		
Fluocinolone	13	0·024	0·024	0·024	Fluocinolone	28.	0·C24	0·024		
acetonide cream	14.	0·025	0·024		acetonide ointment	29.	0·C24	0·023	0·023	
	15.	0·025	0·025		with neomycin	30.	0·C25	0·023	0·025	

The proposed method has been applied to a comprehensive range of accurately compounded samples and the results given in Table 1 demonstrate the wide applicability of the procedure.

The technique incorporates a high degree of specificity and is equally suitable for the examination of samples from both routine manufacture and stability study experiments.

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The semi-quantitative assay of neamine and neomycin C in neomycin by thin-layer chromatography

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Thin-layer chromatographic methods are described for the separation and assay of neamine and neomycin C in neomycin sulphate and its preparations. The samples are chromatographed on binder-free silica gel plates using, as developing solvents, a 3·85% w/v ammonium acetate solution to separate neamine from the neomycins and a 3·4% w/v ammonium hydroxide solution to separate neomycin C from neomycin B and neamine. The spots are made visible by spraying with a 1% v/v solution of t-butyl hypochlorite in dichloroethane-acetic acid (9:1), removing the excess reagent in a stream of cold air and spraying with a 0·5% w/v solution of potassium iodide in 0·5% w/v starch mucilage. The size and intensity of the spots produced is compared with standards and the neamine or neomycin C content of the sample estimated. The results obtained for neomycin C are comparable with those given by chromatography on an ion-exchange resin. A possible explanation is offered for the lack of agreement in the case of neamine. The chromatographic systems described are also applied to paromomycin and shown to separate paromamine, paromomycin I and paromomycin II, not only from each other, but also from neamine and the neomycins.

NEOMYCIN is a mixture of basic, water-soluble antibiotics, produced by the growth of certain strains of *Streptomyces fradiae* in a suitable culture medium. The major active constituent of the mixture is neomycin B, but large amounts of its less active stereoisomer, neomycin C, may also be present, together with small amounts of neamine, a comparatively inactive degradation product of the neomycins. Although differing in microbiological potency, these substances have similar chemical properties; chemical assays of neomycin are therefore unsatisfactory unless preceded by a separation of the mixture into its components.

Chromatographic separations of the neomycin complex have been described by Leach & Teeters (1951), Pan & Dutcher (1956), Kaiser (1963), Brodasky (1963), Maehr & Schaffner (1964) and Inouye & Ogawa (1964). None of these methods is simple, rapid or sensitive enough for routine use and the following thin-layer chromatographic procedures have therefore been developed.

Experimental

Neomycin sulphate was examined by thin-layer chromatography on cellulose, kieselguhr, alumina and silica gel plates in a variety of developing solvents. No single system was found which would separate all the constituents, including unidentified impurities, but a complete separation was obtained on binder-free silica gel using two developing solvents—an ammonium acetate solution to separate neamine and an ammonium hydroxide solution to separate neomycin C.

The compounds were located by heating the plate to remove ammonium compounds and spraying either with a 0·2% w/v ninhydrin solution in methanol, or with t-butyl hypochlorite followed by starch-iodide solution

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(Schwartz & Pallansch, 1958). The second method was preferred because it was the more sensitive, detecting 0·01 μg of neamine.

The pure neomycin B and C standards used in the assay are usually obtained as the bases, whereas the sample is in the form of the sulphate. This difference does not affect the estimation, if the loadings are adjusted to allow for the fact that neomycin sulphate contains only 70% of base.

APPARATUS AND REAGENTS

Neamine base (*Leach & Teeters*, 1951), *neomycin B base and neomycin C base* (*Ford & others*, 1955) were dried *in vacuo* at 50° before use. No significant amount of any impurity was detected by the recommended thin-layer chromatographic methods or by ion-exchange resin chromatography (Maehr & Schaffer, 1964). Neomycin B was 99·6% pure, neomycin C, 98% pure by a paper chromatographic assay (Kaiser, 1963), and neamine, 100% pure by ion-exchange resin chromatography (Maehr & Schaffner, 1964).

t-Butyl hypochlorite reagent. Mix t-butyl hypochlorite (1 ml) with dichloroethane (90 ml) and glacial acetic acid (10 ml). Store in a refrigerator.

Starch-iodide reagent. A solution of potassium iodide (0·5 g) in freshly-prepared starch mucilage B.P. (100 ml).

Standard neamine solutions. Dissolve neomycin B base (70 mg) in water (5 ml). To 0·5 ml aliquots of this solution, add 0, 0·1, 0·2, 0·3, 0·4 and 0·5 ml of a solution of neamine base (2·5 mg) in water (5 ml) and dilute to 1 ml in each case. These standards are equivalent to neomycin sulphate containing 0, 0·5, 1·0, 1·5, 2·0 and 2·5% respectively of neamine.

Standard neomycin C solution. To 0, 0·05, 0·10, 0·15, 0·2, 0·25 and 0·3 ml of a solution of neomycin C base (2 mg) in water (4 ml), add 0·50, 0·475, 0·45, 0·425, 0·40, 0·375 and 0·35 ml respectively of a solution of neomycin B base (4 mg) in water (4 ml) and dilute to 1 ml in each case. These standards are equivalent to neomycin sulphate containing 0, 5, 10, 15, 20, 25 and 30% respectively of neomycin C sulphate.

Developing solvent 1. *Neamine assay.* A freshly-prepared solution of Analar grade ammonium acetate (3·85 g) in water (100 ml). 2. *Neomycin C assay.* Strong solution of ammonia B.P. is assayed (British Pharmacopoeia, 1963) and diluted immediately before use to give a solution containing 3·4% w/v of ammonia.

Thin-layer chromatographic plates. Spread a 0·25 mm layer of Kieselgel H (Merck) on 20 × 20 cm glass plates and activate by heating for 1 hr at 110°.

PROCEDURE FOR NEOMYCIN SULPHATE

Sample solution 1. *Neamine assay.* Dissolve 10 mg of sample in water (1 ml). 2. *Neomycin C assay.* Dissolve 5 mg of sample in water (7 ml).

Method. Line a chromatographic tank with filter paper, add the appropriate developing solvent, saturating the paper lining with the solvent, and allow the tank to equilibrate (1 hr). Spot 1 μl of the sample

solution and of each of the appropriate standard solutions on a thin-layer plate and score the surface of the plate 15 cm from the line of application to limit the extent of the solvent run. Stand the plate in the tank with the silica gel layer facing the paper lining and as close to it as practicable, and allow the solvent to run to the scored line. Remove the plate from the tank and dry in a stream of hot air. Allow the plate to cool and spray uniformly with t-butyl hypochlorite reagent (about 10 ml). Remove the excess reagent by standing the plate in a stream of cold air, until a sprayed portion below the original application line gives little or no blue colour with a spot of the starch-iodide reagent (prolonged exposure to the air stream reduces the intensity of the spots). Finally, spray the plate with starch-iodide reagent and compare the size and intensity of the appropriate spot obtained from the sample with that from the standards.

PROCEDURE FOR NEOMYCIN SULPHATE TABLETS

Shake a quantity of powdered tablets equivalent to 500 mg neomycin sulphate with water (50 ml) and filter (Solution A). Dilute 5 ml of Solution A to 70 ml (Solution B). Use 1 μl of Solution A and 1 μl of Solution B for the assay of neamine and neomycin C respectively.

PROCEDURE FOR CREAMS AND OINTMENTS OF NEOMYCIN SULPHATE

Transfer a quantity of sample equivalent to 10 mg neomycin sulphate to a 10 ml graduated centrifuge tube, add chloroform (5 ml), shake vigorously and centrifuge. Note the volume of any separated aqueous phase and dilute to 2 ml with water. Shake vigorously and recentrifuge to separate the aqueous phase (Solution A). Dilute 1 ml of Solution A to 7 ml (Solution B). Use 2 μl of Solution A for the assay of neamine, comparing the spots obtained with those from 2 μl of a 1 + 1 dilution of each of the standard neamine solutions. Use 1 μl of Solution B for the assay of neomycin C.

Results and discussion

The results obtained when neamine, neomycin B and neomycin C were examined by thin-layer chromatography in the ammonium acetate and ammonium hydroxide systems are given in Table 1. Paromamine, paromomycin I and paromomycin II—the constituents of paromomycin, an antibiotic closely related to neomycin—also separate in the recommended systems and results for these compounds are also quoted.

When samples of neomycin sulphate were examined in the ammonium acetate system, an unidentified impurity was detected on the chromatogram between neamine and the neomycins. This was shown to be active against *Bacillus pumilus* on seeded agar plates (Brodasky, 1963). In the ammonia system, the material gives two partially-separated spots running in front of neamine and it may be a mixture of "low potency neomycins," which are thought to be mono-*N*-acetyl derivatives of the neomycins (Rinehart, 1964).

An ion-exchange column chromatographic assay, in which the eluting fractions are determined with ninhydrin, was used as a reference method (Maehr & Schaffner, 1964, as modified by De Rossi, personal communication). The column and thin-layer assays gave similar results for neomycin C, but for neamine higher results were obtained by ion-exchange chromatography than by the thin-layer method (Table 2). Accordingly,

TABLE 1. RUNNING DISTANCES OF NEOMYCIN AND PAROMOMYCIN COMPOUNDS IN AMMONIUM HYDROXIDE AND AMMONIUM ACETATE SOLUTIONS

Compound	Running distances in mm*			
	Compounds run individually	Compounds run as a mixture		
		(1)	(2)	(3)
	Ammonium hydroxide solution			
Neamine	65–75	63–72	—	60–69†
Neomycin C	61–70	49–58	—	47–55
Neomycin B	51–61	37–46	—	36–45
Paromamine	93–102	—	91–102	92–101
Paromomycin II	85–93	—	80–89	79–88
Paromomycin I	73–82	—	67–76	69–74†
	Ammonium acetate solution			
Neamine	49–59	49–60	—	49–59
Neomycin C	13–23	14–25	—	13–23
Neomycin B	13–24			
Paromamine	78–88	—	78–89	78–88
Paromomycin II	37–46	—	36–46	35–45
Paromomycin I	36–45			

* Distances from the starting line to the rear and front of the spot are given to enable the effectiveness of the separation to be judged. Figures quoted are for loadings of 0·1 µg of each compound in 1 µl water; running distance of solvent front, 15 cm.
† Separation of neamine and paromomycin I incomplete.

the fractions of eluate which contained neamine were examined by thin-layer chromatography in the ammonium acetate system. In addition to neamine, three other compounds were detected, all of which reacted with a ninhydrin reagent spray. They are probably responsible for the high neamine result given by the column assay.

TABLE 2. COMPARISON OF THIN-LAYER AND COLUMN CHROMATOGRAPHIC ASSAYS FOR NEAMINE AND NEOMYCIN C

Sample	Thin-layer assay		Column assay	
	Neamine % (mean and range)	Neomycin C sulphate % (mean and range)	Neamine %	Neomycin C sulphate %
1	Nil	8 (7·5–10·0) [9]*	Nil	8
2	2·5 (2·3–2·5) [4]	15 (12·5–17·5) [8]	2·9	14
3	0·3 (0·25–0·5) [4]	17 (15·0–17·5) [8]	1·9	16
4	0·4 (0·25–0·5) [7]	15 (15·0–17·5) [11]	1·5	13
5	2·0 [4]	19 (15·0–20·0) [10]	3·4	16

* Figure in [ ] is number of assays.

The thin-layer assay procedures were applied to neomycin sulphate and to tablets, creams and ointments prepared from it. Recovery experiments were made by adding known amounts of neamine, neomycin C and neomycin B to blank formulations and submitting them to the assay; complete recoveries of the added neamine and neomycin C were obtained.

The neamine contents of neomycin sulphate (26 samples), neomycin sulphate tablets (13 samples) and neomycin creams and ointments (15 samples) from several manufacturers were determined. None of the samples contained more than 2·5% neamine relative to the neomycin sulphate present and only three samples contained more than 1·5%. The neomycin C contents of the samples were also determined. Between 5% and 30% of the neomycin sulphate content was present as neomycin C sulphate, with most results falling in the range 15% to 25%. Each sample was assayed between four and ten times: replicate results fell within ±25% of the mean.

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A rapid method for the estimation of impurities in saccharin and sodium saccharin

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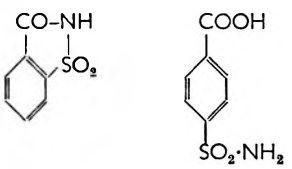
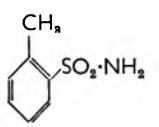
A procedure is described for the detection and estimation of *o*-toluenesulphonamide, saccharin-*o*-toluenesulphonylimide, toluene-2,4-disulphonamide, saccharin-4-sulphonamide, *o*-sulphamoylbenzoic acid, *p*-sulphamoylbenzoic acid and benzoic acid in saccharin and sodium saccharin using thin-layer chromatography on Kieselgel GF_{254}. The solvent system used is chloroform–methanol–strong ammonia solution (100 : 50 : 11·5). Compounds containing a free sulphonamide group are detected by an "*N*-chloro" reaction, while the other compounds are detected by examining the chromatogram in ultraviolet light (253·7 mμ). Impurities in commercial saccharin and sodium saccharin are estimated by comparison on the chromatogram with standards containing purified saccharin or sodium saccharin and suitable amounts of the impurities.

OF the impurities likely to be present in saccharin (I), *p*-sulphamoylbenzoic acid (*p*-acid, II) has received the most attention; the method of the British Pharmacopoeia (1963) for limiting the content of this impurity is a modification of the method suggested by Proctor (1905). It depends on the difference between the titration of total acidity and the titration of ammonia after hydrolysis. Since the method is a difference between two determinations, the results are subject to the errors of both; in addition it is not specific for *p*-acid. Other pharmacopoeias specify a method in which (II), if present, is precipitated when saccharin or sodium saccharin is suitably acidified. This method is not very sensitive.



(I)

(II)



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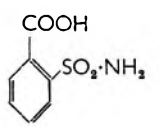
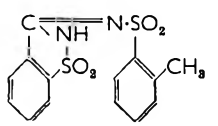


(IV)



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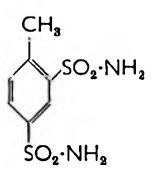
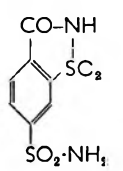


(VI)



(VII)

As most saccharin is manufactured by oxidising *o*-toluenesulphonamide (*o*-amide, III), this is a likely impurity. Richmond & Hill (1919) have suggested an assay procedure in which the saccharin is dissolved in sodium

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bicarbonate solution and (III) extracted with ethyl acetate. The procedure is lengthy and is not specific since other weakly acidic and non-acidic impurities are also extracted by this solvent.

o-Sulphamoylbenzoic acid (*o*-acid, IV) is a less likely impurity: it is hydrolysed by acids to give the ammonium salt of *o*-sulphobenzoic acid and, therefore, if present, it will interfere with the assay procedure of the British Pharmacopoeia (1963). A complex procedure for determining (IV) (Richmond & Hill, 1919) depends on the fact that its solubility in water is relatively high compared with that of saccharin and *p*-acid (II). Although recoveries are reasonable at a 10% level of *o*-acid (IV), the method is quite inadequate for the levels of (IV) normally found in saccharin.

Other impurities have been isolated from saccharin or from the mother liquors during the manufacture of saccharin. These include: saccharin-*o*-toluenesulphonylimide (saccharin-*o*-imide, V) (Klages, 1927) and toluene-2,4-disulphonamide (VI) and saccharin-4-sulphonamide (VII) (Herzog, 1926). No methods have been offered whereby they might be determined in saccharin.

For benzoic and salicylic acids, several pharmacopoeias specify the well known reactions with ferric chloride. Although this reagent is sensitive for salicylic acid, it is not for benzoic acid.

One previous application of chromatography to the determination of *p*-acid (II) and *o*-amide (III) has been described by Franc (1959). This consists of an elaborate method whereby (III) and (II) are nitrated and the nitro-compounds separated by paper chromatography: they are then eluted and determined polarographically.

We have investigated the *direct* separation and estimation of the impurities using thin-layer chromatography.

Experimental

On a thin layer of silica gel using the solvent system chloroform–methanol–strong ammonia solution (100 : 50 : 11·5), saccharin is separated from the impurities benzoic acid and (II) to (VII), but not from salicylic acid. All the impurities were separated from one another, with the exception of *o*-acid (IV) and saccharin-4-sulphonamide (VII).

Small amounts of compounds containing a free sulphonamide group may be detected by a procedure similar to that used by Pan & Dutcher (1956) for the *N*-acetyl derivatives of the neomycin. This involves the formation of *N*-chloro-compounds by spraying with sodium hypochlorite solution, removing the excess hypochlorite by spraying with dextrose solution and finally detecting the *N*-chloro compounds by spraying with a solution of starch and potassium iodide. Saccharin and saccharin-*c*-imide, which do not contain a free sulphonamide group, give a weaker response in this procedure; they and benzoic acid may be detected, however, in ultraviolet light (253·7 mμ) if a suitable phosphor is incorporated in the silica gel. Although *o*-acid (IV) and saccharin-4-amide (VII) have the same Rf value and both are detected by the "*N*-chloro" reaction, only saccharin-4-sulphonamide (VII) is detected in ultraviolet light.

The following method was thus developed and used to estimate the impurities in saccharin and sodium saccharin.

REAGENTS AND MATERIALS

Chromatoplates. Spread a 0·25 mm layer of Kieselgel GF_{254} (Merck) on 20 × 20 cm glass plates, activate by heating for one hr at 110° and store over anhydrous silica gel.

Solvent system. Mix together chloroform (100 vol), methanol (50 vol) and strong ammonia solution B.P. (11·5 vol), all analytical reagent grade. One hr before introducing the chromatoplates, place in a tank lined with filter paper sufficient solvent mixture to form a layer 1·5 cm deep.

Saccharin was recrystallised from water until 750 μg applied to a 0·4 mm layer of Kieselgel GF_{254} showed no detectable impurities when examined by the procedure given below. M.p. 229°. *Sodium saccharin* was recrystallised from 90% v/v ethanol until 750 μg applied to a 0·4 mm layer of Kieselgel GF_{254} showed no detectable impurities when examined by the procedure given below.

o-*Toluenesulphonamide* (*III*) was recrystallised three times from water. M.p. 157·0°. p-*Sulphamoylbenzoic acid* (*II*) was recrystallised three times from water and then from 95% v/v ethanol. M.p. 278·8°. o-*Sulphamoylbenzoic acid* (IV) was made by the method of Richmond & Hill (1919). It had m.p. 153°. *Saccharin*-o-*imide* (*V*) (Klage, 1927) was recrystallised from water. M.p. 255·9°.

Toluene-2,4-*disulphonamide* (VI). Prepare toluene-2,4-disulphonic acid by treating toluene with oleum (20%) at 200–250°. Convert the acid to the dipotassium salt and reflux the salt (65·4 g) for 4 hr with phosphorus pentachloride (166 g) and phosphorus oxychloride (60 ml). Cool and filter off the potassium chloride and excess phosphorus pentachloride. Distil the toluene-2,4-disulphonylchloride at 8 mm Hg (b.p. 204). To the disulphonylchloride (20 g) add water (300 ml) and strong ammonia solution B.P. (25 ml) and stir for 4 hr at 60°. Neutralise with hydrochloric acid and reduce the volume to 150 ml. Filter off the toluene-2,4-disulphonamide and recrystallise from water. M.p. 190°.

Saccharin-4-*sulphonamide* (*VII*). Oxidise toluene-2,4-disulphonamide with alkaline potassium permanganate using the method of Vogel (1956) for the preparation of saccharin from *o*-toluenesulphonamide (III). Recrystallise from water. M.p. 301°.

Benzoic acid of analytical reagent grade.

Strong impurity solution. Dissolve *o*-amide (III) (40 mg), *p*-acid (II) (40 mg), *o*-acid (IV) (20 mg), toluene-2,4-disulphonamide (VI) (20 mg), saccharin-*o*-imide (V) (20 mg) and benzoic acid (100 mg) in methanol–acetone (4:1) (100 ml).

Standard impurity solutions. (*a*) For saccharin: to four 20 ml graduated flasks transfer, respectively, 5, 10, 15 and 20 ml of strong impurity solution. To each flask add 1·0 g purified saccharin, dilute to volume with methanol–acetone (4:1), mix to dissolve the saccharin and adjust to volume if necessary. (*b*) For sodium saccharin: to four 20 ml graduated flasks transfer, respectively, 3·1, 6·25, 9·4 and 12·5 ml of strong impurity solution.

To each flask add 1·25 g purified sodium saccharin, dilute to volume with methanol–acetone (4 : 1), mix to dissolve the sodium saccharin and adjust to volume if necessary.

Sodium hypochlorite solution. Dilute 2 ml of strong sodium hypochlorite solution (containing approximately 16% w/v available chlorine) to 70 ml with water. This solution should be freshly prepared.

Glucose solution. 5% w/v solution in water. This solution should be freshly prepared.

Starch and potassium iodide solution. Mix 50 ml freshly prepared 1% w/v aqueous starch solution with 50 ml 1% w/v aqueous potassium iodide solution and add 1 ml of glacial acetic acid.

PROCEDURE

Dissolve 1·0 g of commercial saccharin or 1·25 g of commercial sodium saccharin in methanol–acetone (4 : 1) and dilute to 20 ml. Apply three 1 μl amounts of the solution to a chromatoplate as a single spot, allowing the solvent to evaporate after each 1 μl application. The spot should be placed on a line approximately 2·5 cm from one edge of the chromatoplate. On the same line apply as single spots three 1 μl amounts of each of the four appropriate standard impurity solutions. The spots should be applied at least 2 cm from either side of the plate and not less than 1·5 cm apart. Score a line across the chromatoplate 10 cm from the line of spots, remove a narrow strip of adsorbent from the sides of the chromatoplate and place the plate in the tank so that the line of spots is a few mm above the solvent surface. When the solvent has ascended as far as the scored line, remove the chromatogram from the tank and dry for 10 min in a stream of warm air.

Examine the chromatogram in ultraviolet light (253·7 mμ) and compare in size and intensity the standard saccharin-*o*-imide and benzoic acid spots with that of any spot from the sample having a similar Rf value.

Heat the chromatogram at 100° (5 min) and while it is still hot, spray with sodium hypochlorite solution until the adsorbent begins to show signs of dampness. Dry (2 min) in a stream of cold air and then spray with glucose solution until the odour of hypochlorous acid is no longer detectable. If the chromatogram has a damp appearance, dry again (2 min) in a stream of cold air. Spray with sufficient starch and potassium iodide solution to render the impurity spots visible. Compare in size and intensity the standard impurity spots with that of any spot from the sample having a corresponding Rf value.

If the amount of an impurity in a sample exceeds that of the highest standard, the procedure should be repeated, taking a reduced amount of sample and adding sufficient purified saccharin or purified sodium saccharin to make the total weight taken equal 1·0 g for saccharin or 1·25 g in the case of sodium saccharin.

If, when the chromatogram is examined in ultraviolet light, an impurity is detected with an Rf value corresponding to *o*-acid (IV), this is probably due to saccharin-4-sulphonamide (VII). Estimate the content of saccharin-4-sulphonamide by repeating the above procedure but replacing the

standards by ones containing saccharin-4-sulphonamide and purified saccharin or sodium saccharin. Estimate the content of *o*-acid by difference.

Results and discussion

The approximate Rf values of the impurities considered are given in Table 1, together with the limits of detection. The latter were determined by chromatographing standard mixtures of purified sodium saccharin with progressively smaller amounts of the impurities. (When purified saccharin was used in preparing the standard mixtures, the Rf values and limits of detection were similar.)

TABLE 1. APPROXIMATE Rf VALUES AND LIMITS OF DETECTION

Compound	Approximate Rf value*	Limits of detection (μg)*	
		"*N*-chloro" reaction	Ultraviolet light (253·7 mμ)
o-Toluenesulphonamide	0·9	0·04	—
Saccharin-*o*-toluenesulphonylimide ..	0·75	—	0·05
Toluene-2,4-disulphonamide	0·65	0·04	—
Saccharin	0·36–0·55†	—	—
Benzoic acid	0·34	—	1
Saccharin-4-sulphonamide	0·27	0·02	0·05
o-Sulphamoylbenzoic acid	0·27	0·02	—
p-Sulphamoylbenzoic acid	0·21	0·02	0·02

* The approximate Rf values and limits of detection of the impurities apply when the impurities are chromatographed in the presence of 187·5 μg of sodium saccharin.

† The Rf values quoted for saccharin are for the rear and front of the spot. All other Rf values are measured from the centre of the spot.

Using the specified standards, there is a good gradation in the size and intensity of the spots for each impurity. The corresponding impurity in a sample of commercial saccharin or sodium saccharin can be placed to the nearest 0·1% in the range 0·1–0·5% or to the nearest 0·2% in the range 0·5–1·0%.

TABLE 2. EXAMINATION OF COMMERCIAL SACCHARIN AND SODIUM SACCHARIN

Sample	% impurity (determined by the proposed chromatographic method)						
	o-Amide (III)	Saccharin-*o*-imide (V)	2,4-Di-amide (VI)	*o*-Acid (IV)	*p*-Acid (II)	Saccharin-4-sulphon-amide (VII)	Benzoic acid
Saccharin A ..	0·7	<0·1	None detected	<0·1	1·0	None detected	None detected
" B ..	0·8	0·6	"	<0·1	0·6	"	"
" C ..	0·8	<0·1	"	0·2	<0·2	"	"
" D ..	0·2	<0·1	"	<0·1	<0·2	"	"
" E ..	0·8	0·1	<0·1	<0·1	<0·2	"	"
" F ..	None detected	None detected	None detected	None detected	None detected	"	"
Sodium saccharin G	<0·1	"	"	<0·05	<0·1	"	"
" " H	0·1	"	"	<0·05	0·1	"	"
" " I	0·1	"	"	<0·05	0·1	"	"
" " J	0·1	"	"	<0·05	1·7	"	"
" " K	0·8	"	"	<0·05	0·2	"	"
" " L	0·3	"	"	0·2	<0·1	"	"

Although the benzoic acid is not detected at such low concentrations as are the other impurities, the above method is a considerable improvement on the pharmacopoeial test. The limit of detection of benzoic acid by the method of the United States Pharmacopeia (1965), for example, is 4%. Salicylic acid is not separated from saccharin by the chromatographic procedure and is not estimated. In this case, however, the method of the U.S.P. (1965) will detect as little as 0·05%.

A number of samples of commercial saccharin and sodium saccharin from various countries were examined by the proposed procedure and the results are shown in Table 2. These results suggest that *o*-amide (III) and *p*-acid (II) are the two impurities that occur in the largest proportion, and that the other impurities considered occur occasionally in small amounts or not at all.

Compared with previously suggested methods, which are often time-consuming or inaccurate, the suggested procedure using thin-layer chromatography offers a rapid and reasonably accurate means of estimating all the impurities considered except salicylic acid.

Acknowledgements. We wish to acknowledge the help of several of our colleagues and, particularly of Mr. P. Oxley for suggesting the use of thin-layer chromatography and of Messrs. R. A. Savidge and S. Duddridge for assistance in the preparation of the impurities.

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SHORT COMMUNICATION

Freeze-dried preparations of *Penicillium spinulosum*

IAN BOYD AND KENNETH BULLOCK

BY a freeze-drying process powders have been obtained containing mould spores in even distribution. These are suitable for use in studying the survival of such spores in the dry state under various environmental conditions, including the presence of fungicidal agents. The preparation of such powders by the more difficult and less easily controlled process of spray-drying has been described by Brown & Bullock (1960).

Spore suspensions were prepared by the following technique. Stock cultures of *Penicillium spinulosum* strain 42237 C.M.I. were maintained on 3% malt extract agar slopes incubated at 25° for 21 days and subsequently stored at 5°. Malt extract agar plate cultures were prepared from a stock slope and incubated at 25° for 21 days; spores were then washed off the surface of the cultures with sterile water. Gentle shaking wetted the large clumps of spores which were pipetted off and discarded. The remaining, as yet unwetted, spores were suspended in a further volume of sterile water by vigorous shaking and the suspension was filtered through a No. 3 sintered glass filter. The final spore suspension contained approximately 85% single spores. Viable counts were performed by the roll-tube method using rose bengal malt extract agar (Brown, 1957).

In the drying process a quantity of spore suspension was added to an aqueous 10% kaolin suspension so that the mixture gave a viable count of about 200×10^3/ml. The mixture (50 ml) was rapidly frozen in a 1 litre round-bottomed flask by rotating in a solid carbon dioxide/acetone mixture. The flask was then attached to a condenser cooled to −72° by solid carbon dioxide and the pressure reduced to 0·1 mm Hg. This primary drying was continued until the temperature of the external surface of the flask rose to 15° (4 hr). The resultant flaky powder was triturated lightly in a mortar and finally dried (16 hr) over phosphorous pentoxide (20–23°/1 mm). Even distribution of spores in the final powder was established by making viable counts on ten replicate 100 mg samples and submitting the results to an analysis of variance ($P = 0{\cdot}50$–$0{\cdot}75$) (Boyd, 1965).

Portions of the powder were stored over phosphorus pentoxide at 5°, 25° and 37°. Samples were removed at intervals, suspended in water and viable counts made on suitable dilutions. The results of one experiment (Fig. 1) show that the spores are obviously sensitive to increased temperature. When the powder was stored at 5° the viable count remained constant up to 40 days.

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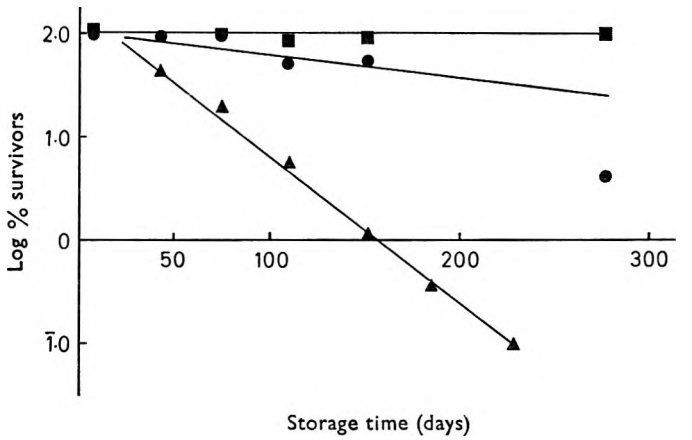

FIG. 1. Effect of temperature of storage on the survival of spores of *P. spinulosum* freeze-dried in kaolin. ■—■ 5°; ●—● 25°; ▲—▲ 37°.

Using the same technique attempts were made to prepare similar spore-containing powders of peptone, malt extract and sodium chloride, but the results were less satisfactory (Table 1). Except for the peptone powder, a smaller percentage of spores survived the drying process and, for some reason at present unknown, replicate counts on these powders during storage showed greater variation than counts on the kaolin powder. The results, however, confirmed that *P. spinulosum* spores die off with increasing rapidity as the storage temperature is increased above 5°.

TABLE 1. THE PERCENTAGE KILL OF SPORES DURING FREEZE-DRYING IN KAOLIN, PEPTONE, MALT EXTRACT AND SODIUM CHLORIDE POWDERS AND THE EFFECT OF STORAGE ON SPORE SURVIVAL, IN THESE POWDERS

Powder	Freeze-drying		Storage			
	% spores killed after		Storage time (days)	% survivors at storage temperatures of		
	Primary drying	Secondary drying		5°	25°	37°
Kaolin	46	49	76	98	95	19
			148	90	56	1
Peptone	20	21	65	74	52	8
			177	59	1	0
Malt extract ..	90	92	56	94	45	34
			156	107	3	0·2
Sodium chloride	88	93	56	101	20	6
			156	3	0	0

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SHORT COMMUNICATION

Germination rate of spores of *Bacillus megaterium*

R. HAMBLETON AND G. J. RIGBY

THE most commonly accepted criteria of bacterial spore germination are loss of heat resistance, acquisition of stainability by simple stains and a change in absorbance (Wynne & Foster, 1948; Powell, 1951; Pulvertaft & Haynes, 1951; Levinson & Sevag, 1953). To these might be added a decrease in resistance to certain germicides. Although it has been suggested that some of these changes occur simultaneously (Campbell, 1957), most of the evidence for this is qualitative rather than quantitative.

This communication deals with a comparison of the rate of loss of refractility observable by phase contrast microscopy, with the rate of loss of heat resistance by a population of *Bacillus megaterium* spores.

METHODS

Spore suspensions of *B. megaterium* ATCC 8245 in dilutions appropriate to the experiment were made. Samples were heat shocked at 80° for 10 min before each experiment and the rate of loss of refractility during incubation was examined by two methods.

Method 1. A loopful of a spore suspension was placed on the surface of a dried MRVP agar (Difco Bacto) plate; the agar layer was uniform and not more than 1 mm thick. A spore coated disc of approximately 3 mm diameter was cut from the plate: it was mounted in a microscope stage designed for the purpose and heated to approximately 37°. A field of 100 or so spores was observed by phase contrast using a × 100 fluorite oil immersion objective. Photographs were taken at intervals during incubation at 37° and counts of the numbers of refractile and non-refractile spores were made from these.

Method 2. A spore suspension (1 ml) was added to double strength MRVP broth (Difco Bacto) (20 ml) and sterile water (19 ml). The whole was shaken at 37° in a water-bath. At the beginning of the experiment and every 5 min a loopful of the culture was removed and placed on the surface of a dried agar plate of Ionagar No. 2 (Oxoid) 1·5% w/v. Discs of approximately 3 mm diameter were cut, mounted on thin slides, covered with thin coverslips and photographed and counted as described above. This method has obvious advantages over the observation and photography of wet preparations.

The rate of loss of heat resistance was examined by incubating spores in liquid MRVP broth as described above (Method 2); a suspension containing about 800,000 viable spores per ml was used. Samples (1·0 ml) were withdrawn from the culture at the beginning of incubation and after 5, 10, 20, 30, 45, 60, 90 and 120 min. Each sample was added to sterile water (9·0 ml) and heated for 10 min at 80° to destroy any

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organisms which had lost this degree of heat resistance; samples were then cooled rapidly. A further dilution (10^{-1}) was made in sterile water, and each of 5 × 1·0 ml samples of this 10^{-2} dilution (containing originally about 200 viable organisms) was plated onto the surface of a dried nutrient agar counting medium. Plates were incubated for 24 hr at 37°.

The number of colonies growing on each plate was counted and by subtraction of the mean count at each time interval from the mean zero count, the mean of the number of spores which had lost heat resistance was calculated.

RESULTS AND DISCUSSION

In all experiments the numbers of viable spores originally present and the numbers which had lost either refractility or heat resistance, were used to calculate weighting coefficients. The figures for % "germination" at each sample time were used to calculate regression equations of probit of % spores germinated on log time. For these calculations, results with probit values lying outside the range 3·7 to 6·3 were rejected unless they lay close to the provisional probit line. Such results are derived from the bottom and from the top of the sigmoidal curve and carry little weight.

From the equations, the slope (b) of each line was calculated and also the log germination time corresponding to probit 5·0 (log G.T.50).

Variances between replicate counts were satisfactory and heterogeneity χ^2 values for the points on each of the lines were not significant.

From the results (Fig. 1) it may be inferred that, in method 2, loss of refractility by some spores occurs within 5 min and continues until about 95% have become phase dark after about 60 min ($b = 2{\cdot}4$).

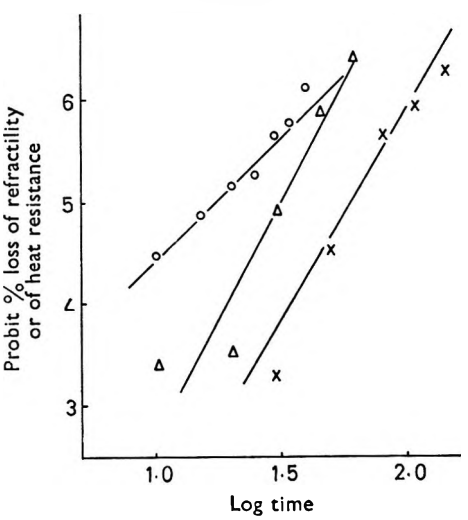

FIG. 1. Regression lines for the probit % loss of refractility on log time. X = method 1 (mean of 6 experiments). ○ = method 2 (mean of 3 experiments). Regression line for the probit % loss of heat resistance on time (△—△) for germinating spores of *B. megaterium* ATCC 8245. (Mean of 3 experiments.)

The results of method 1 suggest a lag of about 40 min during which a very small but increasing proportion of spores lose refractility. During the following 60–70 min the rate of loss of refractility is higher ($b = 4{\cdot}3$). The lag is attributed to slow heat transference from the heated stage through the agar disc to the spores on the surface.

With loss of heat resistance there appears to be a lag of 20–25 min during which the rate of loss of resistance is low. During the subsequent 40 min the rate is high ($b = 4{\cdot}7$).

The G.T.50 values of the two refractility probit lines (17 min and 60 min) differ due to the lag inherent in method 1. The G.T.50 for the heat resistance line is 31 min.

Since the incubation methods used are identical, a comparison can be made between the rate of loss of refractility by method 2 and the rate of loss of heat resistance. The slopes of the lines, 2·4 and 4·7 and the G.T.50 values, 17 min and 31 min differ significantly and suggest that loss of refractility and loss of heat resistance by these spores do not occur simultaneously.

The heated stage (method 1) is a useful method for following rate of loss of refractility. If the lag in heating could be determined with accuracy, so that an arbitrary zero time could be established, the slope of the line would be reduced and might well agree with the slope for method 2.

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The effects of the addition of manganese dioxide to media on the viability of bacteria damaged by x-rays, phenol and radiomimetic agents

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Suspensions of two strains of *Escherichia coli* were counted before and after damage by phenol, X-rays and radiomimetic substances on nutrient agar which had been treated in various ways with manganese dioxide. The medium after some treatments gave increased counts with cells damaged by phenol and dimethyl busulphan and with undamaged cells, the strains responding rather differently. The beneficial action of manganese dioxide was not due to the breakdown of peroxides in the medium and may be due to the adsorption of toxic substances from the medium.

IT is known that manganese dioxide is capable of initiating the breakdown of peroxide in media and its inclusion therefore affords protection towards catalase negative organisms, such as *Shigella dysenteriae* (Proom, Woiwod, Barnes & Orbell, 1950). Richards (1959) claimed that untreated and phenol treated *Escherichia coli* were not influenced significantly by the addition of manganese dioxide to media, and concluded that the inhibitory effects of agar on damaged bacteria were not due to the presence of peroxides therein. However it has now been shown by Board (personal communication) that with phenol-treated *E. coli* toxic effects similar to those reported by Jacobs & Harris (1960, 1961) and by Richards (1959) are due to the presence of peroxides. Board demonstrated a protective effect by including manganese dioxide in a layer of non-nutrient gel over the nutrient agar; the protective effect was not present when manganese dioxide was included in the nutrient agar.

It has been reported that irradiation of cells sensitises them to peroxide produced in the extracellular fluid (Wyss, Clark, Haas & Stone, 1948; Ogg, Adler & Zelle, 1956) and it seemed possible that such cells would also be affected by the presence of peroxides in nutrient media. It was therefore decided to see whether Board's technique would improve media for the recovery of phenol-treated organisms and also to determine what rôle might be played by peroxides in media in the inhibition of irradiated cells and those exposed to radiomimetic substances.

If there were any beneficial effects of including manganese dioxide in media, it would be necessary to ascertain that they were in fact due to decomposition of peroxides. Organisms known to be peroxide sensitive were therefore counted on the various media to determine if manganese dioxide had a favourable effect on their growth.

Experimental

Organisms. Two strains of *Escherichia coli* type 1 (44°+) were used routinely, namely B/r and strain II of Harris, Richards & Whitefield

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(1961). Three other organisms reputed to be sensitive to peroxides were chosen: *Shigella dysenteriae* strain CN 191, obtained from the Wellcome Bacterial collection and described by Proom & others (1950); *Pasteurella boviseptica* strain CN 1066, also obtained from the Wellcome Bacterial collection and recommended by Proom; and *Chromobacterium violaceum* strain B6 of the Department of Agriculture, University of Edinburgh.

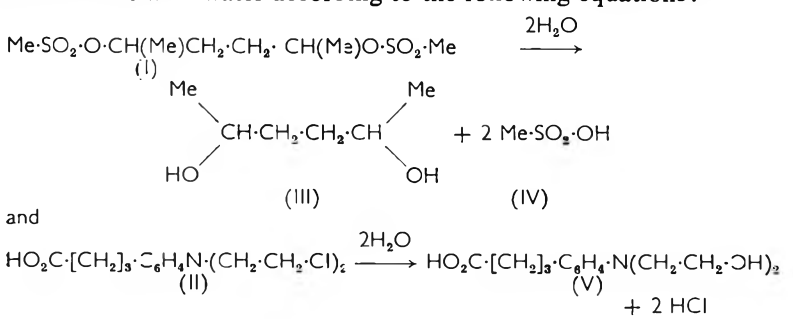
Suspensions. All the organisms were maintained on nutrient agar slopes. A large nutrient agar slope was inoculated from a 24 hr broth culture and the organisms were harvested with 10 ml of glass distilled water after 24 hr at 37°. This dense suspension was washed twice, resuspended in 10 ml of diluent, and stored at 8°. Such suspensions maintain their viability over long periods (Cook & Steel, 1955; Cook, Steel & Wills, 1956) and this was confirmed in the present instance. The suspensions were allowed to age for 5 to 6 days then used over 2 weeks, diluting before each trial, on the basis of an initial nephelometric measurement, to give a concentration of about 2×10^9 viable cells/ml.

BACTERICIDAL TREATMENTS

Phenol. One ml of standardised suspension in glass distilled water was added to 5 ml of phenol solution at 20° ± 0·1 and the mixture was shaken well. The final concentrations of phenol used were 0·65 and 0·90% (w/v) for strains B/r and II respectively, with corresponding reaction times of about 20 and 15 min. These conditions resulted in mortality levels of about 99%, the reaction times being varied to allow for changes in resistance between suspensions.

Irradiations. A Westinghouse Therapy X-ray machine was operated at 220 kV and 15 mA to give doses of about 2500 rad/min in the irradiation cell, as determined by ferrous sulphate dosimetry. Suspensions were diluted in 0·05M phosphate buffer at pH 7·0 before exposure, and were irradiated in shallow layers so that conditions were aerobic. The irradiation times for 99% mortality were 20 min (about 50 Krad) for strain B/r and 12 min (about 30 Krad) for strain II.

Radiomimetic agents. The substances used were dimethyl busulphan (I) and chlorambucil (II; Leukeran, Burroughs Wellcome Ltd.) both of which react with water according to the following equations:



Treatments with dimethyl busulphan were at 37° to keep all the components of the reaction mixture in solution: at 20° insoluble material III

precipitated during the reaction creating difficulties in sampling and introducing indeterminate effects due to adsorption. Precipitation did not occur with chlorambucil and 20° was used with this substance, since it was the same temperature as was used for the phenol treatments. The reactions with water proceed exponentially with time and, under the conditions used, most of the substances had reacted in 1 hr; this was used as the arbitrary contact time to achieve 99% mortality. To obviate frequent weighings of small quantities, 6×10^{-2}M stock solutions in acetone were used. The fact that counts were only slightly lower after 2hr exposure than at 1 hr showed that there was no benefit in prolonging the contact time to permit more complete reaction of the substances and that the acetone itself had no bactericidal action. Since each reaction yields acid products i.e. IV, V and hydrochloric acid, the reactions were performed in buffer solution to prevent adverse pH effects.

Three ml quantities of reaction mixture were formulated as follows: acetone solution of radiomimetic agent, 0·3 to 0·4 ml; standardised bacterial suspensions in buffer, 0·5 ml; phosphate buffer (pH 7·0, 0·05M), to yield 3 ml. Both the suspension and the buffer solutions were equilibrated at 20 or 37° as necessary, before adding the acetone solution of the drug. After 60 min the reaction was quenched by diluting 100 times with buffer solution, further dilutions being made as necessary. The volume of acetone solution of the drug was varied slightly to obtain the required mortality level and this gave concentrations of about 6 to 8×10^{-3}M.

Media. Normal nutrient agar (unlayered control) was prepared from Oxoid CM15 granules (16 g/litre in distilled water) and solidified with 15 g/litre of New Zealand agar, 20 ml quantities being used for the over-dried plates. Four other media were also used. In one, 0·1% manganese dioxide was incorporated into the nutrient agar. The other three comprised normal nutrient agar with a layer of suitably formulated agar gel superimposed on it. These layered media consisted of 15 ml of control medium, but with a nutrient concentration 4/3 of that in the normal medium, covered by a 5 ml layer of gel containing no nutrients, so that the final concentration of nutrients, assuming complete diffusion, was the same as in the non-layered media. The upper layer of one of these three media was a plain agar gel (layered control), that of the second contained 0·1% manganese dioxide and the upper layer of the third contained 0·4% manganese dioxide. In this last medium the amount of manganese dioxide, relative to nutrients and agar, was the same as in the medium comprising normal nutrient agar plus 0·1% of manganese dioxide. The use of layered media was suggested to us by Board, who found them preferable to media containing manganese dioxide dispersed throughout.

To verify the status of the peroxide sensitive organisms, two of the species were also counted on these media in which peroxide formation had been induced by ultraviolet radiation. That such treatment does lead to the formation of peroxides may be inferred from the work of Bacq (1951) and of Latarjet, Caldas, Morenne & Chamaillard (1952) since the indirect lethal action of ultraviolet radiation was alleviated by the presence of catalase. The nutrient agar plates, with lids removed, were irradiated

about 12 inches distant from a Philips 30 W ultraviolet tube and the lids replaced immediately. Suitable exposure times were 15 and 30 min for *Past. boviseptica* and *Shigella dysenteriae* respectively.

Viable counts. Plate counts were made by the surface viable method using 5 replicate 0·017 ml drops and counting after 24 hr at 37°.

Results and discussion

The results of counting untreated cells of both strains of *E. coli* on the five media described above, and after treatment with each of the lethal agents, are given in Table 1. With untreated organisms, only strain B/r grown on the medium layered with 0·4% MnO_2 gel gave counts which the *t*-test showed to be significantly higher than the appropriate control, all other counts showing a non-significant difference from the appropriate control. The results of analyses of variance indicate that there were no significant differences in count between the five media, except in the case of strain II, where the counts were low on two of the modified media.

TABLE 1. THE EFFECT OF INCLUDING MANGANESE DIOXIDE IN THE NUTRIENT AGAR ON THE VIABLE COUNT OF *E. coli*

Treatment	Strain	Proportional count* on			
		Control +0·1% MnO_2	Layered control	Layered control with 0·1% MnO_2	Layered control with 0·4% MnO_2
None	II	82	82	99	85
	B/r	104	129	161	170†
Phenol	II	147†	84	175†	235†
	B/r	297†	39	158†	263†
X-rays	II	49	104	95	115
	B/r	70	100	97	92
Chlorambucil	II	62	90	99	105
	B/r	107	98	102	93
Dimethyl busulphan	II	43†	112	120	139†
	B/r	100	99	136†	168†

* Relative to unlayered control as 100. Mean of 5 trials in all cases except 4 trials with strains B/r and II with X-rays and 6 trials with strain II and dimethyl busulphan.
† Significantly different from the appropriate control (p. 5).

With phenol-treated organisms the strains varied markedly in their responsiveness, and analyses of variance showed that modification of the medium produced significant effects ($P < 0·001$ in all instances). Strain II showed significantly higher counts on all media containing manganese dioxide and the latter was more effective in the layered media than when added to normal medium. Strain B/r behaved in a similar way in that the inclusion of manganese dioxide was always favourable, except that 0·1% manganese dioxide in the medium was as effective as layering the nutrient medium with 0·4% MnO_2 gel. A further point of difference from strain II was that the layered control gave relatively much lower counts than the normal medium. When organisms had been exposed to X-irradiation, the results of *t*-tests indicate that there were no significant differences from the appropriate controls when manganese dioxide was present.

In the main, results with chlorambucil-treated *E. coli* showed that these organisms, like untreated ones, were insensitive to the inclusion of manganese dioxide in the media and this conclusion is supported by the results of *t*-tests.

After treatment with dimethyl busulphan, suspensions were influenced to a greater extent by media modified with manganese dioxide than were cells damaged by chlorambucil. Significant beneficial effects were observed with strain II counted on media layered with 0·4% MnO_2 gel and with strain B/r on media layered with either concentration of manganese dioxide in agar gel.

Thus the inclusion of manganese dioxide in media could result in increased counts of suspensions of *E. coli* after exposure to certain lethal agents. Since the dioxide is known to initiate the decomposition of peroxides, it remained to decide whether the favourable effects were due to this attribute or not. The peroxide-sensitive organisms were therefore counted on the various media to see whether normal medium contained peroxide and, if so, whether manganese dioxide had a favourable effect on the growth of these organisms. The results given in Table 2 showed that in no instance was there a significant improvement in the various media when manganese dioxide was present. Thus the medium employed here would be described by Proom & others (1950) as a "good" medium, i.e. one containing little or no peroxide.

TABLE 2. THE EFFECT OF INCLUDING MANGANESE DIOXIDE IN THE NUTRIENT AGAR ON THE VIABLE COUNTS OF UNTREATED PEROXIDE SENSITIVE BACTERIA

	Proportional count* on			
			Layered control with	
Organism	Control +0·1% MnO_2	Layered control	0·1% MnO_2	0·4% MnO_2
Chromobacterium violaceum	12	94	97	87
Shigella dysenteriae	31	108	143	110
Pasteurella boviseptica ..	76	109	105	102

* Relative to unlayered control as 100. Mean of 5 trials with *Chromobacterium violaceum* and 6 trials with the other two organisms. In no case did the *t*-test indicate that differences from the appropriate control were significant.

TABLE 3. THE EFFECT OF INCLUDING MANGANESE DIOXIDE IN NORMAL AND ULTRAVIOLET IRRADIATED MEDIA ON THE VIABLE COUNTS OF UNTREATED PEROXIDE SENSITIVE BACTERIA

		Proportional count* on				
					Layered control with	
Organism	Medium	Control	Control +0·1% MnO_2	Layered control	0·1% MnO_2	0·4% MnO_2
Shigella dysenteriae	Unirradiated	100	117	89	116	116
	Irradiated	28	117	2	111	117
Pasteurella boviseptica	Unirradiated	100	126	39	123	157
	Irradiated	<2	126	<1	115	132

* Relative to unirradiated normal control as 100, mean of 5 trials.

Finally, it was important to verify that these test organisms were in fact peroxide sensitive. Two of the species, *Shigella dysenteriae* and *Pasteurella boviseptica*, were therefore counted on the same range of media as before and also on these media in which peroxide formation had been induced by ultraviolet radiation. Table 3 presents the results of these experiments. It is obvious that the ultraviolet irradiation of the media resulted in low counts of both species, and that low counts did not occur when manganese dioxide was present, regardless of the manner of its presentation.

It must be concluded that although treatment of the media with manganese dioxide resulted in higher counts in some circumstances, notably after phenol treatment, this beneficial effect was not due to the removal of peroxide from the media. It is possible that the manganese dioxide adsorbed toxic substances, having effects similar to those noted with ferric chloride and activated charcoal (Jacobs & Harris, 1960, 1961).

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SHORT COMMUNICATION

Sizing *Bacillus megaterium* spore populations as a basis for studying their viability

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SEVERAL reports have described microbiological applications of the Coulter Counter, particularly for determining the total count of viable and non-viable vegetative bacteria (Garrett & Miller, 1965) or bacterial spores (Kubitschek, 1958) in suspension. The value of the instrument would be greatly increased if it could be used to differentiate between viable and non-viable bacteria.

Germination of bacterial spores, commonly taken as a criterion of their viability, is accompanied by an increase in cell volume, increases in packed cell volume being as much as 100% during pre-emergence swelling of *Bacillus cereus* and *B. subtilis* spores (Hitchins, Gould & Hurst, 1963). If, during germination, changes in the volume of individual cells are sufficiently large, the Coulter Counter might be employed to enumerate either germinated spores or unchanged spores, and hence yield an estimate of the viability of the original population. The object of the reported work was to recognise the limits of a volume distribution of resting spores of *B. megaterium* and to test whether or not this distribution could be measured in the presence of germinated spores of the same organism.

MATERIALS AND METHODS

The Model B Coulter counter used was fitted with a 30 μ aperture tube and was calibrated with 0·796 μ diameter polystyrene latex spheres (Dow Chemical Company). Size distributions were obtained from a Model J plotter which automatically records particle counts in selected size ranges.

Electrolyte. Aqueous solutions of sodium chloride with concentrations ranging from 0·5 to 10% were tested for instrument sensitivity and count stability; a 2% solution was satisfactory and was used in the reported experiments. It was freshly prepared each day.

Medium. MR–VP granules (Difco) 3·4 g, distilled water to 100 ml; 20 ml volumes were sterilised by autoclaving for 5 min at 121°.

Electrolyte used for counting, distilled water and medium were all filtered through a stack of cellulose ester membranes (Millipore Filter Corporation) consisting of two of mean pore size 0·8 μ on one of 0·22 μ. This treatment gave a stable background count for the electrolyte of less than 1% of the spore count. All glassware was routinely cleaned with "chromic acid mixture" and washed with tap water followed by filtered distilled water; it was then stored in closed containers. Blank determinations showed that sampling and diluting procedures did not increase the background count beyond the arbitrary 1% level.

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Organism. Spores of *Bacillus megaterium* (ATCC 8245) were produced on potato extract agar (Powers, Ehret & Bannon, 1957). Surface growth from six plates was collected in filtered distilled water, washed three times and then suspended in water.

Incubation and sizing. Sufficient stock spore suspension was added to medium maintained at 37° to give a total spore count of about 2×10^7/ml. At intervals, samples (1 ml) were withdrawn and immediately diluted with 19 ml of electrolyte. Volume distributions of particles in these suspensions were plotted between the limits of 0·14 and 0·88 μ^3 within 5 min of their preparation. An aperture current setting of $\frac{1}{2}$ and an amplification setting of $\frac{1}{8}$ were used.

RESULTS AND DISCUSSION

A typical plot of the volume distribution of unincubated spores is shown in Fig. 1a. Counts recorded between 0·14 to 0·26 μ^3 represent

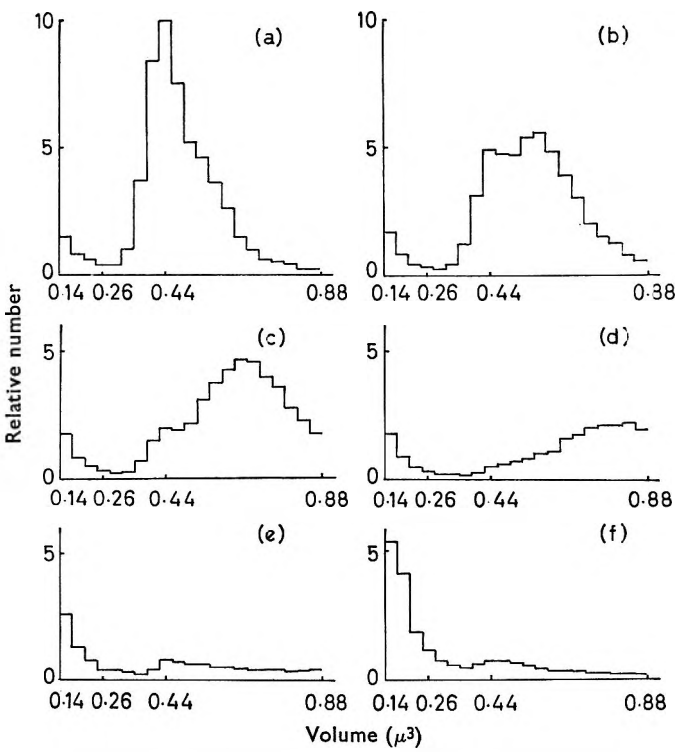

FIG. 1. Effect of incubation in nutrient medium on volume distributions of *B. megaterium* spores. (a) 0 min. (b) 30 min. (c) 45 min. (d) 60 min. (e) 90 min. (f) 120 min.

background count and instrument noise. Above a volume of 0·26 μ^3 the counts show an asymmetric distribution of cell volumes with a single peak lying at about 0·44 μ^3, the count at 0·88 μ^3 being essentially zero. Distributions of cell volumes for spores incubated for increasing periods

in nutrient medium are given in Fig. 1b–f. During incubation cells undergo an asynchronous increase in volume, and after 30 and 45 min incubation bimodal volume distributions are observed (Fig. 1b, c). After 90 min incubation increases are such that more than 85% of the cells have volumes greater than 0·88 μ^3; Fig. 1e shows that the volumes of the remaining 15% are asymmetrically distributed around a peak of 0·44 μ^3 like the unincubated spores. The same peak is seen after 120 min incubation (Fig. 1f), although a greater background count than after 90 min is recorded. With further incubation the background count continues to rise and this is probably caused by cell debris shed during emergence and cell multiplication. The appearance of a constant number of cells with unchanged volumes after incubation for between 90 and 120 min suggests that these cells constitute the inactive fraction of the original spore population. Heating the spore suspension at 65° for 6 hr before 90 min incubation, results in disappearance of the 0·44 μ^3 peak.

These results indicate that estimates of the number of inactive spores in suspension may be made by counting cells within the volume range 0·26 to 0·88 μ^3 after a period of incubation in a suitable medium. To test this, samples of the spore suspension were heated at 90° for graded times (between 4 and 20 min) known from plate counts to yield survival levels ranging from 75 to 30%. These samples were incubated for 90 min and plots of the volume distributions of inactive spores were then made. The distributions were all typical of that for unincubated spores and the areas under the plots increased with heating time as expected from the plate counts. These experiments show that the Model B Coulter counter can be used to count unchanged *B. megaterium* spores in the presence of germinated spores, and that the absence of a change in cell volume on incubation can probably be used as a criterion of spore inactivation. As yet the fate or cells undergoing volume changes is not known.

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SHORT COMMUNICATION

The effect of peptone on the inactivation of a bacteriophage by chemical antimicrobial agents

W. R. L. BROWN* AND A. H. THOMAS

BECAUSE of their intracellular parasitic nature, infectious virus particles are almost invariably closely associated with substances derived from the host organism. In the chemical disinfection of virus contaminated material, the possible interference in the action of the inactivating agent by extraneous organic matter is therefore of particular importance. Little quantitative information on such effects is available however. Peptone reduces the activity of formaldehyde, phenol and sodium hypochlorite against *Bacillus subtilis* spores (Bullock & Rawlins, 1950, 1954) and it has been used here to illustrate the effect of non-viral organic matter on the chemical inactivation of a bacterial virus.

The bacteriophage (coliphage T6r) and the host bacterium (*Escherichia coli*) were cultivated as previously described (Cook & Brown, 1963). Plaque counts were made by the soft agar layer method (Adams, 1959; Brown, Cook & Oduro-Yeboah, 1964). All phage inocula were taken from a single suspension in peptone water (1·0% peptone and 0·5% sodium chloride) containing approximately 1×10^9 plaque forming units (p.f.u.) per ml and stored at 4°.

Oxoid Peptone (L37/1881A) was used throughout, a sterile aqueous solution containing 2·0% peptone and 0·5% sodium chloride being diluted as required with 0·5% aqueous sodium chloride solution. The antimicrobial agents were cetrimide B.P., chloramine-T (B.D.H. Laboratory Reagent), formaldehyde solution (Analar) and phenol (Analar).

Inactivation of the phage by formaldehyde in the presence of 0·01% peptone follows the kinetics of a first order chemical reaction while inactivation by cetrimide and chloramine-T shows deviations from first order kinetics similar, though not identical, to those reported for phenol (Brown, Cook & Oduro-Yeboah, 1965). Details of the time-survivor curves obtained will be published elsewhere. For the present work, concentrations of the antimicrobial agents were used which, in the presence of 0·01% peptone, gave at least 90% inactivation of the inoculum in a fixed time of exposure (30 min) at 25°. The effect of peptone was tested therefore on the resistant fraction of the inoculum which survived the initial rapid inactivation by cetrimide, chloramine-T and phenol.

Reaction mixtures were prepared by adding 0·9 ml peptone solution of appropriate concentration and 0·1 ml phage suspension to 9 ml aqueous solution of the antimicrobial agent. After 30 min at 25°, samples were diluted with a suitable neutralising agent and 6 replicate plates prepared for each dilution. Each test was performed in triplicate. Reaction mixture samples containing phenol were diluted in peptone water; those

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containing cetrimide, chloramine-T and formaldehyde were diluted with peptone water containing 3·0% Tween 80, 1·0% sodium sulphite and 1·0% dimedone-morpholine respectively. Initial dilutions were made at least 1 in 10 and subsequent dilutions, when required, were prepared with peptone water in every case. Except where otherwise indicated, the samples were plated immediately after dilution. This procedure had been shown previously to neutralise effectively the antimicrobial agents.

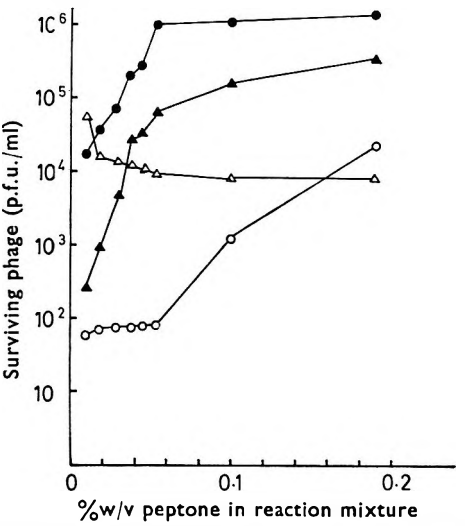

FIG. 1. The effect of peptone concentration on the inactivation of coliphage T6r in 30 min at 25° by cetrimide, $7{\cdot}2 \times 10^{-3}$% w/v ○—○. Chloramine, $3{\cdot}6 \times 10^{-2}$% w/v ▲—▲. Formaldehyde, $3{\cdot}6 \times 10^{-1}$% w/v △—△. Phenol, 2·25% w/v ●—●. Phage inoculum $1{\cdot}68 \times 10^{7}$ plaque forming units (p.f.u.) per ml. Each point represents the mean of 3 replicate tests.

Phage suspensions in 1% and 0·01% peptone solutions without antimicrobial agents, of the same titre as the inocula of the reaction mixtures, showed no change in plaque count during 14 days at 25°.

The effect of peptone concentration on the inactivation of the phage by one concentration of each antimicrobial agent is shown in Fig. 1. The minimum concentration of peptone tested (0·01%) was that resulting from the peptone present in the phage inoculum and, for the present work, was regarded as the control preparation.

Cetrimide, chloramine-T and phenol showed a *decrease* in viricidal activity as the peptone concentration *increased*. The effect on phenol was most pronounced up to 0·055% peptone whereas with cetrimide it became significant only above this concentration. Further work is in progress to elucidate the significance of this concentration of peptone.

Formaldehyde showed an *increase* in activity with increased peptone concentration, an effect which is contrary to the usual effect in the inactivation of bacteria. A possible explanation is suggested by the results obtained when diluted samples of formaldehyde treated phage were stored at 4° and 24° and plaque counts performed at intervals during 5 hr (Fig. 2). The recovery of phage from reaction mixtures containing 0·01% peptone increased markedly with time of storage, the increase being more

pronounced at the higher temperature tested. No such increase in recovery was found in samples from reaction mixtures containing 0·1% peptone. Some of the phage particles treated with formaldehyde in the presence of low peptone concentrations are, therefore, reversibly inactivated as has been reported by Schultz & Gebhardt (1935) for a staphylococcus phage and by Heicken & Spicher (1959) for coliphages. In the presence of higher concentrations of peptone the phage are permanently damaged, possibly by the formation of a formaldehyde-peptone complex which is held at the site of action more firmly than formaldehyde alone.

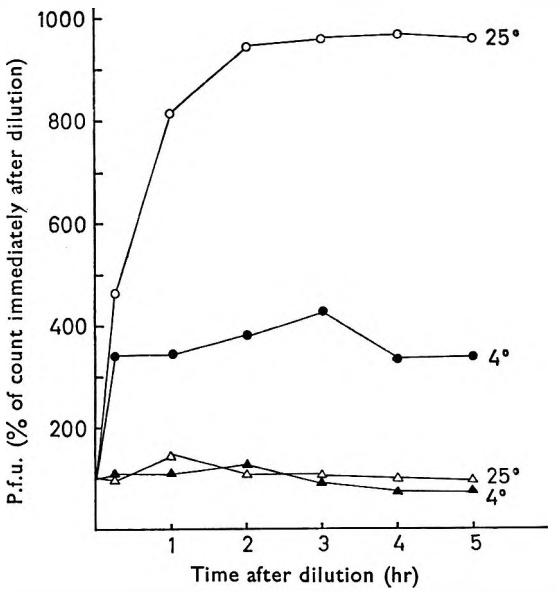

FIG. 2. The recovery of coliphage T6r in 1% dimedone - morpholine at 4° and 24° after treatment with formaldehyde ($3{\cdot}6 \times 10^{-1}$% w/v for 30 min at 25°) in the presence of peptone. Counts expressed as % of count immediately after dilution (ca. 99·9% inactivation of original inoculum). Each point represents the mean of duplicate tests. 0·01% peptone in reaction mixture ○, ●. 0·10% peptone in reaction mixture △, ▲.

Samples from reaction mixtures containing cetrimide, chloramine-T or phenol and 0·01% peptone showed no alteration in recovery with time after dilution.

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The effect of benzoic acid, phenol and hydroxybenzoates on the oxygen uptake and growth of some lipolytic fungi

STELLA M. RIVERS* AND V. WALTERS†

Oxygen uptake by lipolytic fungi on arachis oil is in the order *Aspergillus flavus* (brown) > *A. niger* > *Rhizopus arrhizus* > *Rh. cohnii* > *A. flavus* (green). Respiration is stimulated by low concentrations of benzoic acid, hydroxybenzoates and phenol, but with higher concentrations it is inhibited. Concentrations which give relatively low oxygen uptake are also fungistatic. The factors governing partitioning in oil-water systems are used to calculate the inhibitory concentration of benzoic acid in the aqueous phase.

MICRO-ORGANISMS can multiply in emulsions containing preservatives (Atkins, 1950; Eggins & Walters, 1963). The preservation of emulsions has been reviewed by Wedderburn (1964) and the activity of antibacterials in two-phase systems by Bean, Heman-Ackah & Thomas (1965). We report the effect of toxic agents on the respiration and growth in agar medium of five lipolytic fungi, *Aspergillus flavus* Link (green), *A. flavus* Link (brown), *A. niger*, *Rhizopus arrhizus* Fischer and *Rh. cohnii*.

Experimental

The materials, suspensions containing 150×10^6 *Aspergillus flavus* (brown) spores per ml and 300×10^6 ml^{-1} of the other species, and the method of measuring oxygen uptake (Umbreit, Burris & Stauffer, 1964) were as described by Rivers (1965). Salts solution was a Czapek's solution modified as follows, $NaNO_3$ (0·4%), KH_2PO_4 (0·1%), NaCl (0·1%), $MgSO_4.7H_2O$ (0·1%), $FeSO_4.7H_2O$ (0·002%) in water. The control flasks contained 1·5 ml salts solution, 0·5 ml spore suspension, 0·15 ml arachis oil and water to 3 ml. Reaction flasks included suitable concentrations of benzoic acid A.R., sodium benzoate A.R., methyl hydroxybenzoate B.P., propyl hydroxybenzoate B.P., or phenol A.R., dissolved initially in the oil or salts solution; these substances were also included in the agar culture medium which consisted of salts solution 50%, arachis oil 10%, "Oxoid" agar 2% and water. The oil was added aseptically to the aqueous phase and 20 ml quantities of the well-shaken media poured, immediately before setting, into Petri dishes. The plates were inoculated centrally on their surfaces with one-drop volumes of *Rh. arrhizus* or *A. flavus* (brown) spore suspensions and incubated at 30°. The diameters of the zones of growth were measured after 7 and 14 days. Aseptic precautions and sterile materials were used, where appropriate, throughout.

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Results

The percentages of antifungal agents refer to the overall concentrations unless otherwise stated, and all results are the means of replicate experiments.

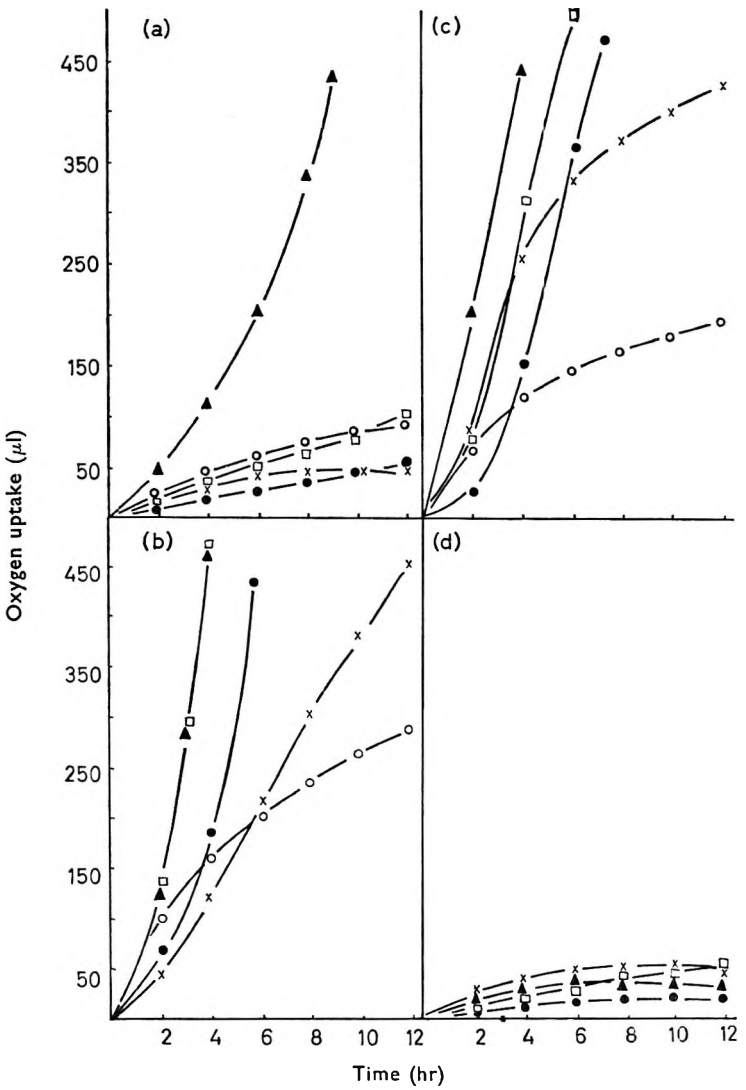

FIG. 1. The effect of (b) 0·1% sodium benzoate, (c) 0·01 and (d) 0·1% benzoic acid cn the oxygen uptake of *A. flavus* (brown) — ▲ — ▲ —. *A. niger* — □ — □ —. *Rh. arrhizus* — ○ — ○ . *Rh. cohnii* — ● — ●—. *A. flavus* (green) — X — X —. *Rh. arrhizus* had no uptake in the presence of 0·1% benzoic acid. Controls (a) contain: 0·15 ml arachis oil, 0·5 ml spore suspension, 1·5 ml salts solution, water to 3 ml. Reaction suspensions include the test substance at overall concentration stated.

Sodium benzoate 0·1% and benzoic acid 0·01% stimulated oxygen uptake, but benzoic acid 0·1% suppressed respiration, immediately with *Rh. arrhizus* and after some hours with the other species except *A. flavus* (green) (Fig. 1). The effect on *Rh. arrhizus* and *A. flavus* (brown) of benzoic acid 0·1%, obtained by dissolving it in either the oil or the aqueous phase, was the same, indicating equilibrium distribution. After 12 hr there were no viable spores remaining in these two reaction mixtures; subsequent inocula in Sabouraud medium gave no growth on incubation at 30°.

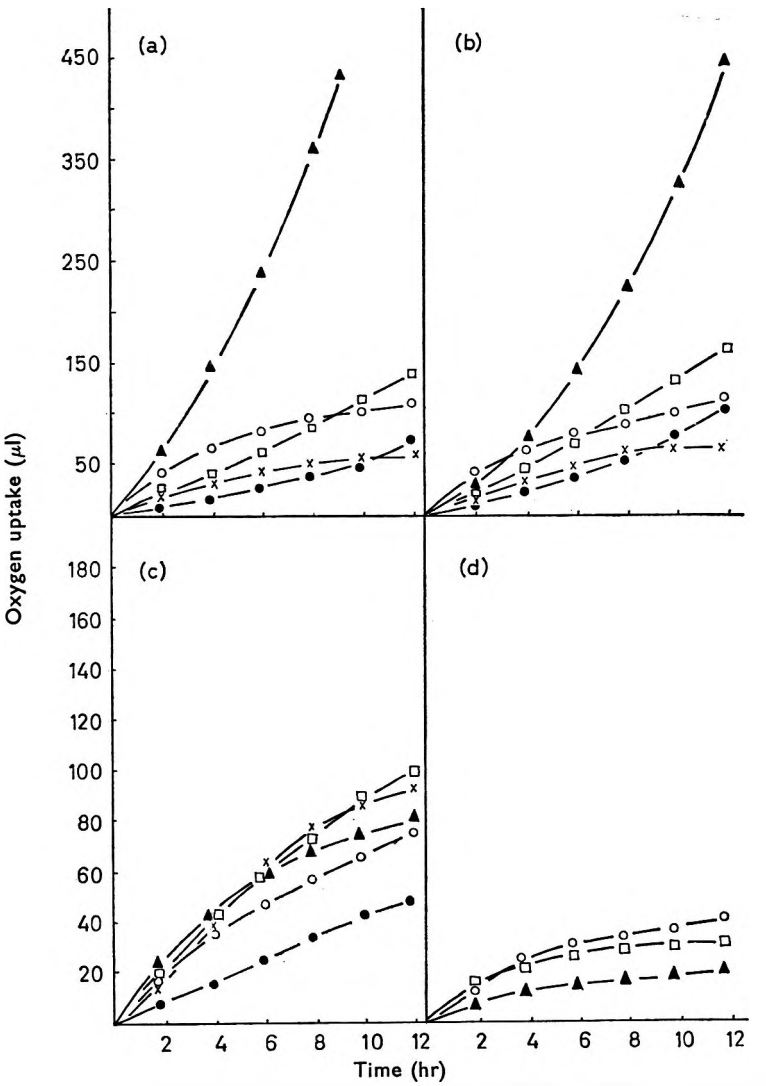

FIG. 2. The effect of concentration of phenol (a) 0·025%, (b) 0·05%, (c) 0·25% and (d) 0·5%, on oxygen uptake. Symbols as in Fig. 1. No experiments were made with *Rh. cohnii* and *A. flavus* (green) with 0·5% phenol.

Phenol in low concentration, 0·025 and 0·05%, had no effect on the oxygen uptake of *Rh. arrhizus* and *A. flavus* (green), whereas that of *Rh. cohnii* and *A. niger* was slightly increased and *A. flavus* (brown) decreased. With 0·25% the uptake of all organisms except *A. flavus* (green) was depressed and especially that of *A. flavus* (brown); the decrease was greater with 0·5% (Fig. 2).

Methyl hydroxybenzoate 0·01% had no effect on *A. flavus* (green and brown) but the other organisms were stimulated as were all with 0·1%; the response with 0·01% was greatest with *A. niger* and *Rh. cohnii*. Propyl hydroxybenzoate 0·01% also had a slight stimulating effect and 0·05% more so, except with *A. flavus* (brown) (Fig. 3). Methyl and propyl hydroxybenzoates, 0·023 and 0·012% (equivalent to the concentrations in Solution for Eye-drops), stimulated the oxygen uptake of *Rh. arrhizus* (from 113 μl in 12 hr for the control to 213 μl) but that of *A. flavus* (brown) was relatively unchanged.

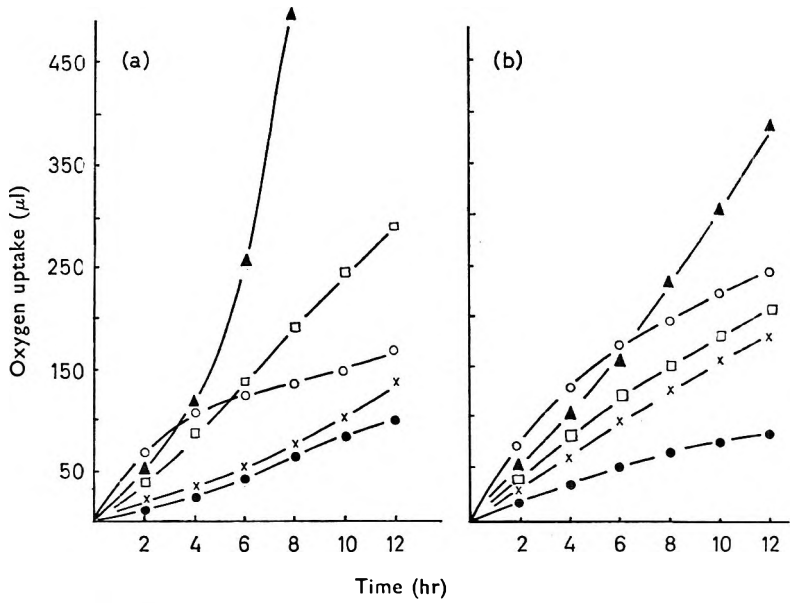

FIG. 3. The effect of (a) methyl hydroxybenzoate, 0·1% and (b) propyl hydroxybenzoate, 0·05%, on oxygen uptake. Symbols as in Fig. 1.

A. flavus (brown) and *Rh. arrhizus* gave visible growth on the culture medium within 1 and 4 days respectively but in the absence of oil there was no growth even after 14 days. Media containing benzoic acid, 0·1 and 0·2%, and phenol, 0·25 and 0·5%, inhibited the growth of both organisms. *Rh. arrhizus* was more susceptible, since its growth was also inhibited by benzoic acid 0·02%, and methyl hydroxybenzoate 0·1% (Table 1). Normal metabolism was affected by propyl hydroxybenzoate and phenol since the mycelia, where growth occurred, were more felted and sporulation was retarded.

TABLE 1. EFFECT OF ANTIFUNGAL AGENTS ON COLONY GROWTH AND ON OXYGEN UPTAKE OF SPORES

Organism	Antifungal agent	Overall concentration %	Colony diameter mm after:		Oxygen uptake µl in 12 hr (or stated hr)
			7 days	14 days	
A. flavus (brown)	Benzoic acid	0·01	56	71·5	467 (5 hr)
		0·02	54	74	—
		0·1	0	0	23
		0·2	0	0	—
" "	Methyl hydroxy-benzoate	0·01	57	67	420 (10 hr)
		0·02	53	64	—
		0·1	39	60	506 (8 hr)
" "	Propyl hydroxy-benzoate	0·01	55	68	426 (8 hr)
		0·02	52	64	—
		0·05	38	60	389
" "	Methyl and propyl hydroxy-benzoate	0·023 and 0·012	54	68	524 (7 hr)
" "	Phenol	0·05	38	72	445
		0·1	14	27	—
		0·25	0	0	83
		0·5	0	0	21
" "	Controls: with oil	0	55	75	471 (7 hr)
	without oil	0	0	0	81
Rh. arrhizus	Benzoic acid	0·01	17	38	220
		0·02	0	0	—
		0·1	0	0	0
		0·2	0	0	—
" "	Methyl hydroxy-benzoate	0·01	16	37	149
		0·02	11	33	—
		0·1	0	0	164
" "	Propyl hydroxy-benzoate	0·01	15	38	160
		0·02	13	34	—
		0·05	5	16	245
" "	Methyl and propyl hydroxy-benzoate	0·023 and 0·012	6	27	213
" "	Phenol	0·05	7	24	112
		0·1	4	5	—
		0·25	0	0	75
		0·5	0	0	41
" "	Controls: with oil	0	17	30	111
	without oil	0	0	0	60

Colony diameters and oxygen uptake are means of results of replicate plates and reaction flasks at 30°
Agar culture medium: 2 ml arachis oil and 18 ml inorganic salts-agar, inoculated on the surface with one drop of spore suspension.
Oxygen uptake reaction mixtures: 0·15 ml arachis oil, 0·5 ml spore suspension, 1·5 ml salts solution, water to 3 ml.
Antifungal agents at overall concentrations given were included.

Discussion

When arachis oil was used as the sole carbon source, the oxygen uptake of the fungi increased in the order, *A. flavus* (green), *Rh. cohnii*, *Rh. arrhizus*, *A. niger* and *A. flavus* (brown).

Stimulation of respiration occurred with low concentrations of antifungal agents, presumably due to their metabolism, but higher concentrations were inhibitory. A similar effect has been noted with a variety of toxic substances and organisms (Owens, 1953; Simon, 1953; McCallan, Miller & Weed, 1954; Beveridge & Hugo, 1964; Smith & Shennan, 1966).

The preservative action of organic acids is due mainly to the unionised molecules (Rahn & Conn, 1944; Aalto, Firman & Rigler, 1953; Bandelin, 1958; Albert, 1965; Winsley & Walters, 1965), and the concentration of undissociated acid in the aqueous phase may be calculated by the method outlined by Martin (1960).

For benzoic acid 0·1%, the pH of the aqueous phase of the oxygen uptake reaction mixture was 3·4, $K_a = 6{\cdot}4 \times 10^{-5}$ and assuming the oil/water partition coefficient, k = 5·3 (Garrett & Woods, 1953), the calculated equilibrium concentration of unionised acid in the aqueous phase, $[HA]_w = 0{\cdot}073\%$. This is a concentration which inhibited oxygen uptake, growth in agar and was fungicidal in 12 hr. That this should be so is not surprising, since unionised benzoic acid at a concentration of about 0·02% has been shown to inhibit the growth of *A. niger* (Evans & Dunbar, 1965; Winsley & Walters, 1965) and at concentrations >0·025% to inhibit the growth of *Saccharomyces ellipsoideus* (Rahn & Conn, 1944). With sodium benzoate 0·1%, the calculated value of $[HA]_w$ is 0·007%. At such low concentrations of unionised benzoic acid no growth inhibition occurs, but respiration is stimulated and presumably the unionised molecules or anions are metabolised. Thus with *A. flavus* (brown) the oxygen uptake of the control was 410 μl in 8 hr and that of a reaction mixture in which oil was replaced by sodium benzoate, 552 μl in 8 hr, but when both oil and sodium benzoate were present the oxygen uptake increased to 466 μl in 4 hr.

Species difference between the fungi was well shown by their response to phenol. *A. flavus* (brown) had the greatest oxygen uptake on arachis oil. It was also the most susceptible organism to phenol which has an arachis oil/water partition coefficient of about 5·6 (Bean & others, 1965). This greater sensitivity was evident also with the oil-soluble propyl hydroxybenzoate. The aqueous concentrations of hydroxybenzoates were, however, much less than those of 0·4% methyl and 0·1% propyl hydroxybenzoate which inhibited the respiration of yeast (Wailes, 1962) and of 0·1 and 0·03% respectively, which inhibited the growth of *A. niger* (Bandelin, 1958).

Although conditions between the manometric and culture experiments were not identical, some degree of correlation existed (Table 1). For example, benzoic acid 0·1% inhibited the growth of *Rh. arrhizus* and *A. flavus* (brown) and there was no oxygen uptake with *Rh. arrhizus* nor after about 6 hr with *A. flavus* (brown). These results, like others (Wailes, 1962; Chauhan, Rivers & Walters, 1963), indicate that oxygen uptake is much reduced when there is fungistasis; it ceases only in fungicidal conditions.

Before germination, spores swell due to water intake. Swelling is accompanied by a large increase in oxygen uptake and requires a utilisable exogenous carbon source to provide energy for the increase in plasticity of the spore wall (Ekundayo & Carlile, 1964; Ekundayo, 1966; Marchant & White, 1966). The permeability of the spore wall is also increased; thus Caltrider & Gottlieb (1963) and Chauhan & others (1963) found that spores were more sensitive to toxic agents after 4–6 hr in nutrient media

when they were swollen or producing germ-tubes. With organisms like *A. flavus* (brown) which are able to utilise oil, the effect of preservatives does not seem to be entirely dependent upon the concentration in the aqueous phase. There would appear to be an advantage in the use of a preservative with an oil/water partition coefficient sufficient to produce a suitable concentration in the oil phase. Additionally, for ionisable compounds, the pH of the aqueous phase should be such as to ensure that the biologically active form is mainly present.

Within the limitations of the need ultimately to test a preservative in the emulsion or cream in which it is to be used, simulated manometric conditions can provide activity values rapidly and with ease.

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The stimulation of strychnine metabolism in rats by some anticonvulsant compounds

J. F. HOWES AND W. H. HUNTER

The metabolism of a series of 4-acetyl-1-naphthyl ethers has been examined in rats and in fractions of rat liver homogenate. The administration of the compounds to rats increases the rate of strychnine metabolism when the compounds used are metabolised by the microsomal fraction of liver homogenate.

THE stimulation of oxidative metabolism of foreign compounds by prior administration of drugs is now a well-recognised phenomenon (Gillette, 1963) and may influence the results of accepted pharmacological screening techniques. We wish to report the increase in strychnine metabolism produced by prior administration to rats of some anticonvulsant compounds (Hunter, Quinton, Sherman, Worthing & Boscott, 1964). We have also studied the oxidation of the compounds themselves *in vivo*, and *in vitro* using homogenate of rat liver and fractions derived therefrom by centrifuging.

Experimental

MATERIALS AND METHODS

The compounds were all prepared from 4-acetyl-1-naphthol as described by Hunter & others (1964) and are listed in Table 1 (R = 4-acetyl-1-naphthyl throughout). They were administered intraperitoneally, as suspensions in 1 ml arachis oil, at 250 mg/kg to male Wistar rats weighing about 300 g. Male rats were used because they metabolise strychnine more rapidly than do females (Kato, Chiesara & Vassanelli, 1962). Forty eight hr after this single dose the animals were killed, the livers were removed and covered with ice-cold isotonic potassium chloride solution. The livers were homogenised with 2 volumes of isotonic potassium chloride solution in a slow speed Waring blender (LO setting for 15 sec). The homogenates were centrifuged for 30 min at 10,000 *g* to remove nuclei, mitochondria and cell debris leaving the supernatant fraction. This supernatant fraction, consisting of microsomes and soluble fraction, was used to determine the rates of strychnine metabolism. In addition, the supernatant was further separated into a microsome fraction and a soluble fraction, used separately for some experiments as described below. The supernatant, prepared as described, was centrifuged 1 hr at 140,000 *g* at 0–4° and the soluble fraction removed. This soluble fraction was adjusted to pH 9·4 with glycine–sodium hydroxide buffer (Sorensen's glycine II) and to this was added NAD (2 μ mole/5 ml of solution).

The microsome pellet was resuspended in the original volume of phosphate buffer (pH 7·4) to give the microsome fraction.

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TABLE 1. THE RATE OF METABOLISM OF STRYCHNINE BY SUPERNATANT FRACTION OF RAT LIVER (R = 4-ACETYL-1-NAPHTHYL)

Compound administered to rats		μMoles strychnine metabolised/g liver/hr
None		0·23 (0·22–0·24)
R–O·CH_2·CO·Me	I	0·62 (0·58–0·64)
R–O·CH_2·CHOH·Me	II	0·45 (0·44–0·47)
R–O·CH_2·CHOH·CH_2·Me	III	0·45 (0·40–0·48)
R–O·CH_2·CHOH·CH_2·O·$CHMe_2$	IV	0·53 (0·50–0·55)
R–O·CH_2·CH_2·OH	V	0·25 (0·22–0·28)
R–O·CH_2·CH_2·O·Me	VI	0·57 (0·55–0·60)

Compounds administered at 250 mg/kg i.p. in arachis oil.
Control animals received only arachis oil.
Each result represents the average of at least six animals.
Figures in brackets are the range of results.

METABOLISM OF STRYCHNINE *in vitro*

The supernatant (2 ml) was mixed with a solution (0·5 ml) containing nicotinamide (50 μmole), Mg^{++} (75 μmole), K^+ (100 μmole) and strychnine sulphate (432 μg; 1 μmole). To this was added a reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system consisting of glucose-6-phosphate (20 μmole), NADP (0·4 μmole) and glucose-6-phosphate dehydrogenase (present in liver supernatant fraction). The mixed solution was made up to 5·0 ml with 0·05M "Tris" buffer to a final pH of 8·2. All pH values were checked using a Pye "Dynacap" pH meter and the solutions shaken (1 hr) at 37°. Aliquots (2 ml) of the incubation mixture were treated with sodium chloride (2 g), N sodium hydroxide (1 ml) and extracted with 1·5% v/v solution of isoamyl alcohol (25 ml) in heptane for 45 min. The solutions were centrifuged, the organic layer (20 ml) re-extracted with 0·1N hydrochloric acid (4 ml) and the absorbance of this acid extract was determined at 254 mμ and 278 mμ. This procedure gave strychnine recoveries corresponding to 98–102%. Chromatography on thin-layer plates showed that strychnine metabolites were not extracted with the strychnine.

METABOLISM OF THE COMPOUNDS *in vitro*

For the experiments on the *in vitro* metabolism of the compounds, colloidal suspensions were prepared by suspending the solid materials (12 μmole) in phosphate buffer (pH 7·4; 1·0 ml) and subjecting them to ultrasonic disintegration with an "Electrosonic" 80 Kc/sec ultrasonic generator for 1 min. Samples of the compounds prepared in this way were examined by thin-layer chromatography on silica-gel to verify that the procedure did not affect the compounds chemically. The colloidal suspensions (1 ml) were added to the appropriate liver fractions (5 ml) prepared as described above. When the supernatant fraction or microsome suspensions were used, the flasks were shaken for 1 hr at 37°. For the soluble fraction the flasks were incubated for 1 hr at 25°, without shaking. At the end of the incubation period, protein was precipitated by adding saturated barium hydroxide solution (2 ml) followed by zinc sulphate solution (2 ml, 20% w/v) and the precipitate was removed by centrifuging (5 min at 5,000 *g*). The clear solutions were decanted and

extracted with peroxide-free ether (3 × 5 ml); the extracts were evaporated *in vacuo* at room temperature and the residues examined by thin layer chromatography (Table 2).

TABLE 2. COLOUR REACTIONS AND Rf VALUES OF COMPOUNDS AND THEIR METABOLITES ON THIN-LAYER CHROMATOGRAMS (R = 4-ACETYL-1-NAPHTHYL)

Compound		Rf values in solvents A, B and C			Colour reactions with reagents			
		A	B	C	D	E	F	G
R–O·CH_2·CO·Me	I	0·90	0·75	0·70	g	—	—	y
R–O·CH_2·CHOH·Me	II	0·81	0·64	0·62	,,	—	—	—
R–O·CH_2·CHOH·CH_2·Me	III	0·83	0·68	0·64	,,	—	—	—
R–O·CH_2·CO·CH_2·Me		0·93	0·69	0·73	,,	—	—	y
R–O·CH_2·CHOH·CH_2·O·$CHMe_2$	IV	0·78	0·71	0·65	,,	—	—	—
R–O·CH_2·CH_2·OH	V	0·72	0·53	0·50	,,	—	—	—
R–O·CH_2·CH_2·O·Me	VI	0·81	0·67	0·67	,,	—	—	—
R–OH		0·67	0·75	0·61	g–b	y	o	—
R–O·CH_2·CO_2H		0·28	0·10	0·10	g	y	—	—
R–O·CH_2·CHOH·CH_2OH		0·34	0·35	0·20	,,	—	—	—
VII (Metabolite of III)		0·35	0·30	0·22	,,	—	—	—

Silica gel (Merck G) plates were used 250μ × 22 cm × 22 cm.
Solvents A Chloroform - methanol 9:1 v/v.
B Benzene - ethyl acetate 2:3 v/v.
C Chloroform - acetone 5:4 v/v.
Fresh solvent was used for each plate and allowed to run 10–12 cm.
Reagents: D Ferric chloride - perchloric acid.
E Acridine 0·1% w/v in absolute ethanol.
F Diazotised *p*-nitroaniline.
G 2,4-Dinitrophenylhydrazine 0·1% w/v in ethanol.
g—green; b—blue; y—yellow; o—orange.

The formaldehyde produced during the oxidative removal of the methyl group from 2-(4-acetyl-1-naphthoxy)ethyl methyl ether VI was determined by the method of Nash (1953) as modified by Cochin & Axelrod (1959).

METABOLISM OF THE COMPOUNDS *in vivo*

The fate *in vivo* of the compounds was examined by administering them to male rats as described above; control animals received arachis oil only. Urine was collected for 48 hr after dosage, from both treated and control animals. Pooled urine samples from pairs of rats receiving the same compound were extracted with n-butanol to remove the metabolites and, if present, unchanged compounds. The butanol was removed *in vacuo* at room temperature and small samples of the extracts examined by chromatography on silica gel plates (250 μ, Merck, silica gel G) using benzene-ethyl acetate (2:3 v/v) as developing solvent. The extracts were then hydrolysed by heating (1 hr) with 2N hydrochloric acid at 100°. (The use of bacterial β-glucuronidase was less successful). The hydrolysed materials were extracted with n-butanol or ether, and the metabolites separated by thin layer chromatography on silica gel plates (250 μ; Merck, silica gel G.). They were identified by comparing their chromatographic behaviour with that of authentic standards and by their colour reactions with ferric chloride-perchloric acid solution (20% w/w hydrated ferric chloride in perchloric acid), with 2,4-dinitrophenylhydrazine (0·1% w/v in ethanol), and with diazotised *p*-nitroaniline (Bray, Thorpe & White, 1950). A solution of acridine (0·1% w/v in absolute ethanol) was used to locate acidic metabolites (LeHongre, Tanner & Rentschler, 1957).

The colour reactions and Rf values of the compounds and their metabolic products are shown in Table 2. Acidic metabolites were further characterised by chromatography on pH gradient plates (Shellard & Alam, personal communication).

4-Acetyl-1-naphthol, when present as a metabolite was identified also by the characteristic bathochromic shift in the ultraviolet spectrum in basic solution (Fig. 1).

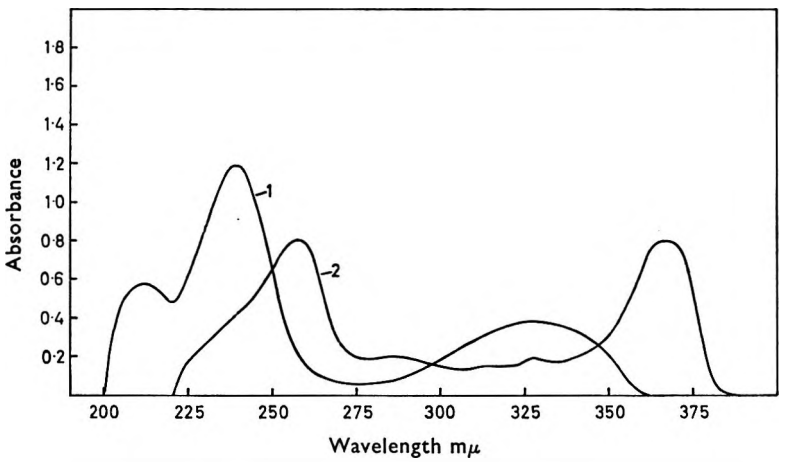

FIG. 1. Ultraviolet spectrum of 4-acetyl-1-naphthol. Curve 1. Solution of 5 μg/ml in 70% ethanol (Spectroscopic grade). Curve 2. The same solution with 1 drop of 1·0N NaOH added. On addition of acid, curve 1 is reproduced.

Results

The rates of strychnine metabolism in liver supernatant fraction from control rats and from treated rats are recorded in Table 1. With the exception of the primary alcohol (V) the compounds effectively stimulated

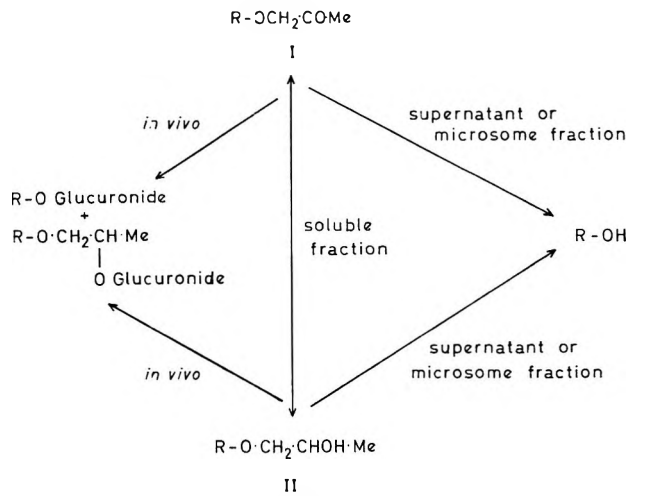

FIG. 2. Metabolic pathway for compounds I and II.

strychnine metabolism in rat liver. In Figs 2–4 are shown the products of the metabolism of the compounds used, *in vivo*, in liver supernatant fraction, in microsome suspensions and in the soluble fraction. In the series of compounds studied, the metabolic pathway *in vivo* could be demonstrated qualitatively at least, *in vitro*, by using a suitable fraction of liver homogenate. The *in vivo* metabolism of the ether (VI) led to

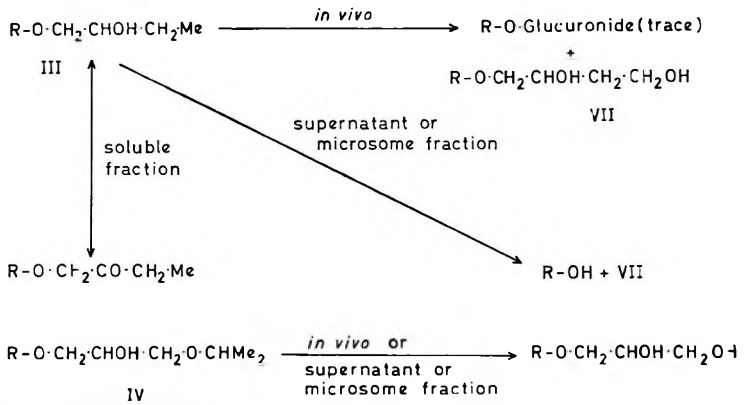

FIG. 3. Metabolic pathway for compounds III and IV.

excretion of the alcohol (V) as the glucuronide (VIII) and, when incubated with liver supernatant fraction or with a microsome suspension, the ether formed 0·33 μmoles of formaldehyde (0·30–0·38 in six experiments) per μmole of the compound. The ether was recovered unchanged after incubation with the soluble fraction of liver homogenate and no formaldehyde was formed.

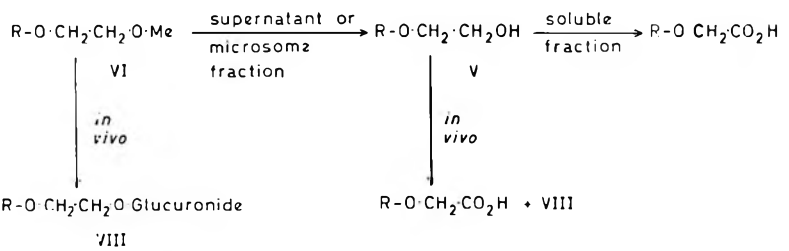

FIG. 4. Metabolic pathway for compounds V and VI.

Discussion

The effect of some anticonvulsant drugs upon the metabolism and toxicity of strychnine has been reported by Kato & others (1962). Oxidative metabolism of strychnine has been shown to occur in the microsomal fraction of liver from various species (Adamson & Fouts, 1959) and 2-hydroxystrychnine is a metabolite in rabbit liver (Tsukamoto, Oguri, Watabe & Yoshimura, 1964). All the compounds that stimulated strychnine metabolism were themselves oxidised by the microsome

fraction. The *in vivo* oxidation of the compounds led to metabolites identical with those produced *in vitro* by microsomes thus demonstrating the essential similarity of the two types of metabolism. Microsomal oxidation of these compounds in the rat could therefore be the process leading to increased rate of strychnine detoxication.

The primary alcohol (V) was the only compound whose *in vivo* metabolic pathway could be reproduced *in vitro* by the soluble fraction of liver homogenate. This alcohol was the only compound that failed to stimulate metabolism of strychnine. The conversion of the alcohol to the methyl ether (VI) produced a potent stimulator of strychnine metabolism from a compound that was initially inactive in this respect. Unlike the alcohol, the ether was oxidised only in the microsome fraction.

The results suggest that the long duration of anti-strychnine activity in this series (Hunter & others, 1964) could be explained by the increased rate of strychnine metabolism. In this series of compounds, only those members that are metabolised in the microsome fraction stimulated strychnine metabolism.

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Some studies of the diffusion of sodium ions through protein solutions and phospholipid sols

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Diffusion of sodium-22 in solutions containing sodium chloride, calcium chloride, bovine plasma albumin, lecithin and cholesterol has been examined by two methods which were modifications of the open-end capillary technique of Anderson & Saddington (1949) and the continuous monitoring technique of Mills & Godbole (1958). Preliminary investigations of semi-infinite systems consisting of lecithin sols layered on albumin solutions have been made. The activity in the capillary at various times during diffusion has been compared with the theoretical activity and good agreement has been found.

THE diffusion of components present in aqueous solutions of large molecules has received but little attention so far. Wang (1954) found that the self-diffusion coefficient of water in ovalbumin solutions was less than in water itself and he was able to obtain information about the shape and hydration of the protein molecules. Other authors (Brady & Salley, 1948; Huinzenga, Grieger & Wall, 1950; Dux & Steigman, 1958, 1959; Clifford & Pethica, 1964) have used radioisotope techniques to study the diffusion of ions in detergents and synthetic ion-exchange resins. Using a conductimetric method, Saunders (1963) has investigated the diffusion of electrolytes (NaCl and $CaCl_2$) in aqueous phospholipid sols containing cholesterol.

In the present paper tracer methods for studying diffusion in aqueous albumin solutions and lecithin sols are described. The effect of concentration of solute and of the addition of calcium chloride and cholesterol is reported. Preliminary investigations of composite systems consisting of lecithin sols layered on albumin solutions are also discussed. By postulating that the bovine plasma albumin layer is semi-infinite and the phospholipid layer finite, and by analogy with conduction of heat in solids, equations have been derived from which it is possible to calculate the concentration of radioisotope in the layers at any given time during the diffusion experiment.

Two experimental techniques have been used: (i) the open-end capillary method of Anderson & Saddington (1949) and (ii) a modification of the continuous monitoring method of Mills (Mills & Godbole, 1958).

Theory

The diffusion of a particle in a system where there is no chemical potential gradient and in which the movement of the particle is due to random molecular motion, is termed self-diffusion. The usual equations describing self-diffusion have been used and modified to satisfy the boundary conditions. Some of our experiments did not strictly follow the conditions for self-diffusion and this is pointed out where applicable.

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The active material was contained initially in a short length of capillary tube, sealed at one end. Diffusion out of the tube took place at the open end into a stirred bath of inactive solution of the same ionic concentration as that present in the tube. The stirring ensured that the activity at the open end of the capillary was zero throughout the experiment, in accordance with the boundary condition.

The transport of material by diffusion is defined by Fick's law:

$$\left(\frac{\partial^2 C}{\partial x^2}\right)_t = \frac{1}{D}\left(\frac{\partial C}{\partial t}\right)_x \quad .. \quad .. \quad .. \quad (1)$$

where C = concentration of solute in any plane, x, from the boundary, t = time during which diffusion has been taking place, D = diffusion coefficient of the solute.

The partial differential equation (1) was solved to satisfy the particular initial and boundary conditions under which the experiment was conducted. An expression was found for the activity, C(x,t), at a plane, x, in the tube after a given time. Integration over the whole length of the capillary yielded an equation for the total activity, $C_{(tot)}$ remaining in the capillary at time, t.

Various mathematical techniques were applied in the derivation of the equations given below and reference was made to Crank (1956), Carslaw & Jaeger (1959) and Jost (1960). The Laplace transformation method was of particular use in the solution of equations applicable to the more complicated systems, e.g., composite semi-infinite media.

1. *Solution of equation* (1) *for a semi-infinite medium*

Initial conditions: $C(x,t) = C(x,0),\ x>0,\ t = 0.$

Boundary conditions: $\left.\begin{aligned} C(x,t) &= C(x,0),\ x = \infty \\ C(x,t) &= 0,\qquad x = 0 \end{aligned}\right\} t > 0.$

The solution for the activity, C(x,t), complying with the initial and boundary conditions was:

$$C(x,t) = C(x,0)\ erf\ \frac{x}{2\sqrt{Dt}} \quad .. \quad .. \quad (2)$$

Integration of equation (2) over the length of the capillary, i.e.,

$$\int_{x=0}^{x=} C(x,t).dx$$

gave

$$C_{(tot)} = C_0 - \frac{2C_0\sqrt{Dt}}{l\sqrt{\pi}} + \frac{C_0\sqrt{Dt}}{l}\ 2\ ierfc\ \frac{l}{2\sqrt{Dt}} \quad .. \quad (3)$$

where C_0 = total initial activity in the capillary of length, *l*.

2. *Solutions for the finite medium*

Initial conditions: $C(x,t) = C(x,0),\ 0<x<l,\ t = 0.$

$$\text{Boundary conditions: } \left.\begin{aligned} C(x,t) &= 0, \; x = 0 \\ \frac{\partial c}{\partial x} &= 0, \; x = l \end{aligned}\right\} \; t > 0.$$

Solving the partial differential equation (1) for the above conditions gave equations (4) and (5) for the activity at plane, x, in the tube. The final equations (6) and (7) were obtained by integration of (4) and (5) respectively.

$$C(x,t) = C(x,0) - C(x,0)\sum_{n=0}^{\infty}(-1)^n\left\{erfc\,\frac{(2nl+x)}{2\sqrt{Dt}} + erfc\,\frac{2(n-1)l-x}{2\sqrt{Dt}}\right\} \quad .. \quad .. \quad .. \quad (4)$$

$$C(x,t) = \frac{4C(x,0)}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^n}{2n+1}\exp\left[-\frac{D(2n+1)^2\pi^2t}{4l^2}\right]\sin\frac{(2n+1)\pi x}{2l} \quad .. \quad .. \quad .. \quad (5)$$

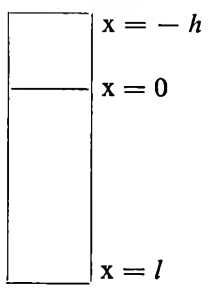
$$C_{(tot)} = C_0 - \frac{2C_0\sqrt{Dt}}{l}\left\{\pi^{-1/2} - \sum_{n=0}^{\infty}(-1)^n\,2\,ierfc\,\frac{nl}{\sqrt{Dt}}\right\} \quad .. \quad (6)$$

$$C_{(tot)} = \frac{8C_0}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\exp\left[-\frac{D(2n+1)^2\pi^2t}{4l^2}\right] \quad .. \quad .. \quad (7)$$

The series converge satisfactorily if equation (6) is used for calculations in which the time of diffusion, t, is small and equation (7) for moderate and long times.

3. *Semi-infinite composite medium*

The composite system discussed in this paper consisted of two layers (I and II). The upper one (I) was treated as a finite medium while the lower one (II) was considered semi-infinite.



The differential equations to be solved were

$$\left(\frac{\partial^2 C_1}{\partial x^2}\right)_t - \frac{1}{D_1}\left(\frac{\partial C_1}{\partial t}\right)_x = 0, \quad -h<x<0, \quad t>0, \quad .. \quad (8)$$

$$\left(\frac{\partial^2 C_2}{\partial x^2}\right)_t - \frac{1}{D_2}\left(\frac{\partial C_2}{\partial t}\right)_x = 0, \quad l>x>0, \quad t>0, \quad .. \quad (9)$$

where C_1 and C_2 are the concentrations at a plane, x; D_1 and D_2 are the diffusion coefficient, of sodium-22 in layers I and II respectively.

Equations (8) and (9) were solved to conform with the following conditions:

Initial condition C_1 and $C_2 = C(x,0)$, $\quad -h<x<\infty \quad t = 0$

and assuming that there is no resistance to diffusion at the interface, $x = 0$, the boundary conditions are:

$$\left.\begin{aligned} &\left.\begin{aligned} C_1(0,t) &= C_2(0,t) \\ D_1\frac{\partial C_1}{\partial x} &= D_2\frac{\partial C_2}{\partial x} \end{aligned}\right\} x = 0, \\ &C_1(x,t) = 0, \qquad x = -h, \\ &C_2(x,t) = C(x,0), \qquad x = \infty, \end{aligned}\right\} t>0$$

By applying the Laplace transformation method the following solutions were obtained:

$$C_1(x,t) = C(x,0) - C(x,0)\sum_{n=0}^{\infty}\alpha^n\left\{erfc\,\frac{(2n+1)h + x}{2\sqrt{D_1 t}} - \alpha\, erfc\,\frac{(2n+1)h - x}{2\sqrt{D_1 t}}\right\} \quad .. \quad (10)$$

$$C_2(x,t) = C(x,0) - \frac{2C(x,0)}{1+\sigma}\sum_{n=0}^{\infty}\alpha^n\, erfc\,\frac{(2n+1)h + kx}{2\sqrt{D_1 t}} \quad .. \quad (11)$$

where $k = \left(\frac{D_1}{D_2}\right)^{1/2}$, $\sigma = \left(\frac{D_2}{D_1}\right)^{1/2}$, $\alpha = \frac{\sigma - 1}{\sigma + 1}$

Expressions for total activity in the capillary after time, t, were obtained by integrating the Laplace transforms of equations (10) and (11) and then applying the Inversion Theorem.

$$C_{1(tot)} = {}^{I}C_0 + \frac{{}^{I}C_0\sqrt{Dt}}{h}\sum_{n=0}^{\infty}\alpha^n\left\{2\, ierfc\,\frac{(2n+1)h}{2\sqrt{D_1 t}} + \alpha\, 2\, ierfc\,\frac{(2n+1)h}{2\sqrt{D_1 t}} - 2\, ierfc\,\frac{nh}{\sqrt{D_1 t}} - \alpha\, 2\, ierfc\,\frac{(n+1)h}{\sqrt{D_1 t}}\right\} \quad .. \quad .. \quad (12)$$

$$C_{2\,(tot)} = {}^{II}C_0 - \frac{{}^{II}C_0\sqrt{D_2 t}}{l(1+\sigma)}\sum_{n=0}^{\infty}\alpha^n\left\{2\, ierfc\,\frac{(2n+1)h}{2\sqrt{D_1 t}} - 2\, ierfc\,\frac{(2n+1)h + kl}{2\sqrt{D_1 t}}\right\} \quad .. \quad .. \quad (13)$$

$$C_{(tot)} = C_{1\,(tot)} + C_{2\,(tot)} \quad .. \quad .. \quad (14)$$

where l = length of infinite layer, $C_{1\,(tot)}$ and $C_{2\,(tot)}$ = activity in layers I and II respectively after time, t, ${}^{I}C_0$ and ${}^{II}C_0$ = initial total activity in layers I and II respectively, i.e., $C_0 = {}^{I}C_0 + {}^{II}C_0$.

Alternative solutions of equations (8) and (9) can be obtained by contour integration. These contain an integral which must be evaluated numerically. This method is particularly useful for systems which are more complicated than that described in this section, e.g. systems with a resistance to diffusion at the interface or a system consisting of three layers. In these cases the expansions of the exponential functions of the Laplace transforms become more difficult to handle so that it is not easy to obtain error-function solutions. Work is proceeding on equations describing the flow of sodium-22 in these systems.

Experimental

METHOD (*a*)

A modification of the open-end capillary technique of Anderson & Saddington (1949); (Wang, 1951; Wang & Miller, 1952; Mills & Kennedy, 1953; Mills & Adamson, 1955). A three-necked flask of one litre capacity held the inactive solution and was immersed in a thermostat bath at 25° ± 0·01° (see Fig. 1). The centre neck of the flask was used to accommodate a mercury-seal stirrer and the capillaries, containing the active solution were placed in the flask on small perspex holders attached to rods through the side necks. The capillaries were made from precision bore Pyrex glass tubing (trade name "Uniform," and distributed by Jencons, Hemel Hempstead, Herts), and were 2–4 cm long with an

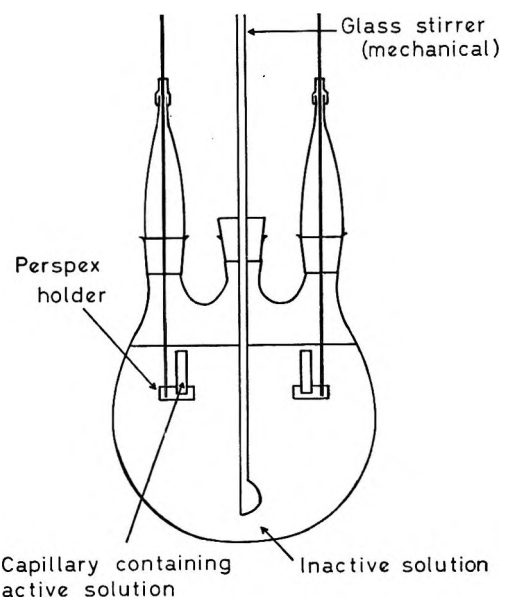


FIG. 1. Apparatus for open-end capillary technique. Method (*a*).

internal diameter of 0·08 cm. One end of the capillary was sealed with a small globule of glass, and the other end was ground flat to promote streamline flow of liquid across the top of the capillary, thus reducing the scooping out of active solution by inactive solution as it flows over the surface. Also to reduce this error, excess active liquid was placed on top of the capillary before immersion in the inactive solution. The capillary internally and externally was coated with a 2% w/w solution of dimethyldichlorosilane in carbon tetrachloride to reduce to a minimum the adsorption of radioactive sodium (Mills & Kennedy, 1953) onto the surface of the capillary. As recommended by other workers (loc. cit.) the stirring rate was approximately 60 rpm. The rate of flow across the open end of the capillary is critical and should be just sufficient to keep the concentration of active ion zero at this point in accordance with the requirements of the mathematical theory. For a more detailed discussion of errors reference should be made to the literature (Wang, 1951; Mills & Kennedy, 1953; Berne & Berggren, 1960).

The lengths of the capillaries were measured with a metal plunger attached to a micrometer: this was the most precise way of obtaining these measurements ($\pm$0·0005 cm).

The solutions were prepared as described below and sufficient radioactive sodium added to give initial counts of approximately 500–1500 cps. The volume of active solution added was taken as negligible and the solutions were not degassed. Capillaries were filled by means of a fine glass pipette and the initial activity determined in a scintillation counter using a sodium iodide well crystal. Counts were accurate to $\pm$0·1% ($P = 0{\cdot}68$). When thermal equilibrium had been established the capillary was immersed completely in the solution in the flask so that the open end was a few millimetres below the surface of the inactive solution. Diffusion took place for a time sufficiently long for the condition

$$\frac{Dt}{l^2} > 0{\cdot}24$$

to be obeyed thus ensuring the validity of equation (7). At the end of the run the capillary was withdrawn and the remaining activity determined.

METHOD (*b*)

Apparatus for continuous monitoring (Mills & Godbole, 1958, 1959). The apparatus was made of brass protected by a non-corrosive paint and held 5½ litres of inactive solution. It was surrounded by a thermostat bath which was maintained at 25° $\pm$ 0·01°. The capillary was placed in a perspex holder in the centre of the sodium iodide well crystal (see Fig. 2). The crystal was shielded against radiation from external sources and from the bath solution by a ring of lead 2 cm thick. To improve the flow of solution over the open end of the capillary a separate compartment containing the stirrer was constructed and the solution circulated through holes in the vertical and horizontal partitions. The rate of stirring was approximately 70 rpm and was controlled by a constant speed device. The capillary was made as in method (*a*) approximately

2 cm long and 0·08 cm diameter and was filled as described previously. In most experiments the initial activity, C_0, was determined because this quantity was used in some of the later calculations. The capillary was then immersed in the inactive solution and the activity remaining in the capillary was counted at intervals; the middle of the count was taken as the corresponding time, t, at which the measurement was made. Diffusion was allowed to proceed for about five days, the capillary was then removed and the background determined: that this was constant throughout the runs was checked by removing the capillary at definite time intervals and counting the residual activity in the bath. After an initial sharp increase the background counting rate was practically constant, an observation in agreement with Mills & Godbole (1958, 1959).

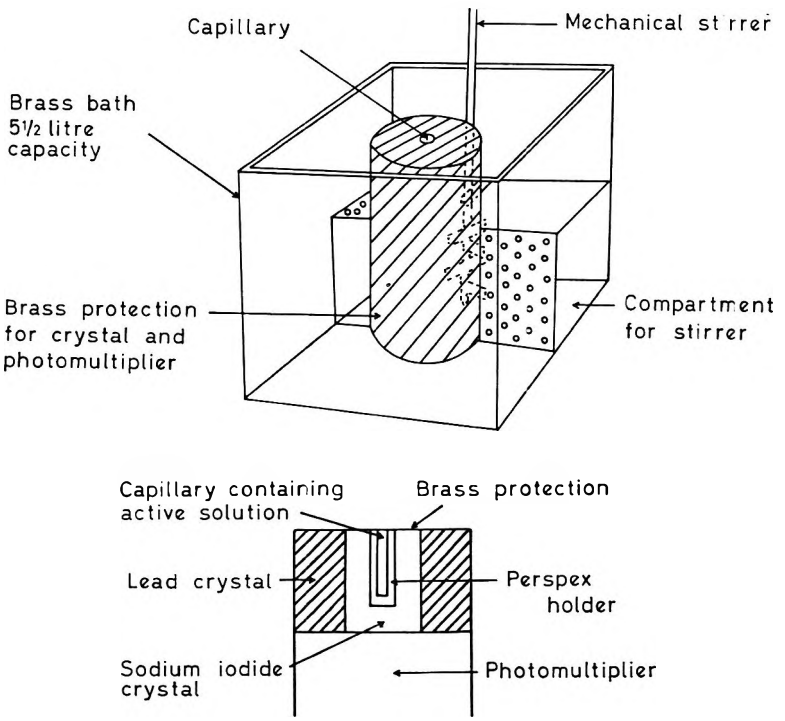


FIG. 2. Apparatus for continuous monitoring. Method (*b*). The lower diagram shows a section through the capillary holder.

PREPARATION OF ALBUMIN SOLUTIONS

A weighed amount of bovine plasma albumin (Fraction V from Bovine Plasma prepared by Armour Pharmaceutical Company Ltd.) was dissolved in boiled and cooled distilled water. Ion-exchange resin (Amberlite Monobed Resin MB-1) kept in methanol (approximately 500 mg) was added and shaken in the albumin solution for 15 min, by which time removal of small electrolytes was assumed to be complete; the resin was

then removed by centrifuging. The required quantities of sodium chloride and calcium chloride were added and the solution made up to weight with water.

PREPARATION OF LECITHIN SOL

Commercial material (A. Merck, Darmstadt) was purified as described by Attwood (1965) and was stored as a solution in chloroform. A suitable quantity of this was evaporated *in vacuo* to constant weight and dissolved in ether together with cholesterol when required. A known volume of boiled, cooled distilled water was added; the ether was removed *in vacuo*, warming when necessary. Nitrogen was bubbled (10 min) through the sol, which was then subjected to ultrasonic irradiation (20 kilocycles/sec for 1–3 hr). The vessel containing the sol was surrounded by ice and water. Ion-exchange resin was added (as for the albumin solutions) and shaken in the sol. The sol was centrifuged to remove the resin together with any titanium deposited in the solution from the probe of the ultrasonic irradiator. The required amounts of calcium chloride, sodium chloride and water were added. Since this caused coagulation of the lecithin-cholesterol micelles the sol was again subjected to ultrasonic irradiation for about 30 min and centrifuged once more.

CALCULATIONS

Diffusion coefficients were calculated from equation (7) which became: (for $t > 140{,}000$ sec).

$$D = \frac{9{\cdot}212\, l^2}{\pi^2 t} \log \left[\frac{8C_0}{\pi^2 C_{(tot)}} \right] \qquad .. \quad .. \quad (15)$$

For method (*a*) this involved a direct substitution of experimental values and a short calculation. To obtain the diffusion coefficients by method (*b*) a plot of log $C_{(tot)}$ against time, t, was made, and the slope of the straight line was calculated by a least squaring procedure. D was found using the equation

$$\text{Slope} = \frac{D\pi^2}{9{\cdot}212\, l^2} \qquad .. \quad .. \quad .. \quad (16)$$

When applying the equations containing the error function terms (equations 3, 12, 13 and 14) it was simpler to calculate $C_{(tot)}$ and to compare this with the experimental result at a given time, t, using values for the diffusion coefficient obtained by equations (15) and (16).

Results

Diffusion of sodium-22 was investigated in solutions containing sodium chloride, calcium chloride, albumin, lecithin and cholesterol. In the composite systems the lower layer (II) consisted of an albumin solution and the upper layer (I) of a lecithin-cholesterol sol.

Tracer diffusion coefficients for sodium-22 in solutions of sodium chloride are listed in Table 1 and for other systems in Table 2. Each result is the mean of at least six measurements except where indicated by the figures in brackets.

Calculated and experimental values for $C_{(tot)}$ are compared in Table 3–5. The percentage difference was evaluated by the following equation:

$$\% \text{ difference} = \frac{\text{difference between calculated and experimental values for } C_{(tot)}}{\text{experimental value for } C_{(tot)}} \times 100 \quad .. \quad (17)$$

TABLE 1. TRACER DIFFUSION COEFFICIENTS FOR SODIUM-22 IN 0·1M AND 0·01M SODIUM CHLORIDE SOLUTIONS

Concentration NaCl mole litre^{-1}	Method	D × 10^5 cm^2 sec^{-1} ± standard deviation (P = 0·68)	
		with stirring	without stirring
0·1	a	$1{\cdot}25_5 \pm 0{\cdot}03$	$1{\cdot}24_9 \pm 0{\cdot}01$
0·1	b	$1{\cdot}27_9 \pm 0{\cdot}01$	—
0·01	a	$1{\cdot}28_3 \pm 0{\cdot}07$ (2)	$1{\cdot}27_6 \pm 0{\cdot}03$
0·01	b	1·306 (1)	—

TABLE 2. TRACER DIFFUSION COEFFICIENTS OF SODIUM-22 IN VARIOUS SYSTEMS

Experiment No.	Method	Composition of solutions		D × 10^5 cm^2 sec^{-1} ± standard deviation (P = 0·68)
		in capillary	in bath	
1	a	0·1M NaCl 10% BPA	0·1M NaCl 10% BPA	$1{\cdot}11_7 \pm 0{\cdot}08$
2	a	0·1M NaCl 0·001M $CaCl_2$ 10% BPA	0·1M NaCl 0·001M $CaCl_2$ 10% BPA	$1{\cdot}13_6 \pm 0{\cdot}08$
3	a	0·1M NaCl 0·001M $CaCl_2$	0·1M NaCl 0·001M $CaCl_2$	$1{\cdot}20_8 \pm 0{\cdot}04$
4	b	0·1M NaCl 2·5% BPA	0·1M NaCl	$1{\cdot}24_4$ (1)
5	b	0·1M NaCl 5% BPA	0·1M NaCl	$1{\cdot}23_6 \pm 0{\cdot}09$
6	b	0·1M NaCl 7·5% BPA	0·1M NaCl	$1{\cdot}20_3$ (1)
7	b	0·1M NaCl 10% BPA	0·1M NaCl	$1{\cdot}07_0$ (1)
8	a	0·1M NaCl 10% BPA	0·1M NaCl	$1{\cdot}13_8 \pm 0{\cdot}05$
	a	0·1M NaCl 0·001M $CaCl_2$ 10% BPA	0·1M NaCl 0·001M $CaCl_2$	$1{\cdot}10_8 \pm 0{\cdot}07$
10	b	0·1M NaCl 0·001M $CaCl_2$ 10% BPA	0·1M NaCl 0·001M $CaCl_2$	$1{\cdot}11_8 \pm 0{\cdot}01$ (2)
11	a	0·1M NaCl 5% lecithin	0·1M NaCl	$1{\cdot}17_5 \pm 0{\cdot}05$
12	b	0·1M NaCl 5% lecithin	0·1M NaCl	$1{\cdot}20_8 \pm 0{\cdot}05$
13	a	0·1M NaCl 0·001M $CaCl_2$ 10% lecithin 5% cholesterol	0·1M NaCl 0·001M $CaCl_2$	$0{\cdot}70_1 \pm 0{\cdot}03$
14	a	"	"	$0{\cdot}92_8 \pm 0{\cdot}01$
15	b	"	"	$0{\cdot}94_0 \pm 0{\cdot}04$

All solutions of bovine plasma albumin (BPA), lecithin and cholesterol were % w/w
Results (13) and (14) different batches of lecithin.

Discussion

CALIBRATION OF THE APPARATUS AND COMPARISON OF METHODS (*a*) AND (*b*)

To ensure that the correct stirring speeds were being used in both types of apparatus (*a*) and (*b*), diffusion runs were made with sodium-22 in solutions of sodium chloride at various strengths. The self-diffusion coefficients thus obtained were compared with those in the literature.

TABLE 3. COMPARISON OF EXPERIMENTAL AND CALCULATED VALUES OF $C_{(tot)}$ FOR DIFFUSION OF SODIUM-22 IN 0·1M SODIUM CHLORIDE AQUEOUS SOLUTIONS WHEN THE SYSTEM IS SEMI-INFINITE
[Experiments 1, 2 and 3 by method (*a*). Experiment 4 by method (*b*)]

Experiment No.	Time of diffusion (sec)	C(tot) (experimental) ±0·1%	C(tot) (calculated)	Difference %
1	19,680	471·0	478·4	1·6
2	63,720	331·8	342·7	3·7
3	68,640	707·1	711·0	0·6
4	3,652	981·5	985·9	0·4
,,	6,670	944·4	941·8	0·2
,,	10,648	906·5	897·6	1·0
,,	15,045	874·7	857·2	2·0
,,	17,698	852·4	836·7	1·8
,,	73,786	559·3	577·4	3·2

This is shown graphically in Fig. 3 where D is plotted against $\sqrt{C}$ together with the results from other authors (Nielson, Adamson & Cobble, 1952; Wang & Miller, 1952; Mills & Adamson, 1955; Mills & Godbole, 1960). The deviation about the mean is indicated for our results and for those from the literature which were measured at the same concentrations.

TABLE 4. COMPARISON OF EXPERIMENTAL AND CALCULATED VALUES OF $C_{(tot)}$ FOR DIFFUSION OF SODIUM-22 IN 0·1M SODIUM CHLORIDE AND 0·001M CALCIUM CHLORIDE AQUEOUS SOLUTIONS CONTAINING (1) 10% W/W ALBUMIN OR (2 AND 3) 10% W/W LECITHIN AND 5% W/W CHOLESTEROL WHEN THE SYSTEM IS SEMI-INFINITE USING CONTINUOUS MONITORING METHOD (*b*)
[Results (2) and (3) from different sols]

Experiment No.	Time of diffusion (sec)	C(tot) (experimental)	C(tot) (calculated)	Difference %
	3,010	883·8	882·6	0·1
1	60,740	596·7	579·6	2·9
	75,238	545·0	531·9	2·4
	4,251	567·7	561·2	1·1
2	58,467	406·8	391·7	3·7
	85,802	355·0	347·1	2·2
	2,454	597·0	590·5	1·0
	5,631	575·3	563·7	2·0
3	75,495	370·7	365·1	1·2
	86,210	351·6	350·1	0·4
	93,518	339·6	234·7	1·5

TABLE 5. COMPARISON OF EXPERIMENTAL AND CALCULATED VALUES OF $C_{(tot)}$ FOR THE DIFFUSION OF SODIUM-22 IN SEMI-INFINITE COMPOSITE SYSTEMS FROM TWO EXPERIMENTS

Experiment No.	Time of diffusion (sec)	C(tot) (experimental)	C(tot) (calculated)	Difference %
	4,670	605·3	603·6	0·39
	7,864	594·1	582·7	1·45
1	12,748	575·6	562·2	2·10
	14,373	571·6	555·7	2·94
	20,997	554·0	532·5	3·88
	3,241	1,316	1,332	1·23
	6,796	1,262	1,269	6·55
2	10,587	1,224	1,227	0·21
	16,422	1,175	1,174	0·94
	25,742	1,115	1,107	0·72

Using method (*b*) (continuous monitor) our results are in good agreement with those of Mills & Godbole (1958), who found that the self-diffusion coefficient of the sodium ion in 0·1M sodium chloride was $1{\cdot}277 \pm 0{\cdot}002_3 \times 10^{-5}$ cm^{2}sec^{-1}. The standard deviation of the measurements reported in this paper at the same concentration was slightly larger. The correct stirring speed is important when using method (*b*) and when this was reduced from 72 rpm to 50 rpm the diffusion coefficient fell from 1·28 to $1{\cdot}26 \times 10^5$ cm^{2}sec^{-1} at 0·1M sodium chloride.

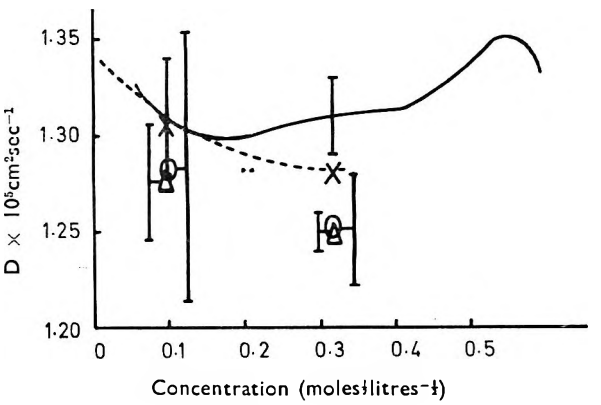


FIG. 3. Graph of D × 10^5 cm^2 sec^{-1} of sodium-22 against concentration (moles½ litre$^{-½}$) for solutions of sodium chloride. The accuracy of the results from method (a) is indicated. 1. Open-end capillary (Wang & Miller 1952). 2. Continuous monitoring (Mills & Godbole 1960). 3. × no stirring; ○ stirring; △ continuous monitoring. See Table 1.

All the results obtained by method (*a*) were slightly lower than those quoted in the literature although all likely sources of error had been reduced to a minimum. There is a wide variation in the results reported by different authors and no completely satisfactory explanation has been given. Maintaining the correct stirring speed does not appear to be critical with method (*a*) and this is in accordance with the conclusion reached by Clifford & Pethica (1964).

Method (*a*) is relatively simple and the time of a diffusion experiment is shorter than in method (*b*). In addition, several tubes can be immersed in the inactive solution at the same time, thus enabling a number of simultaneous results to be obtained. It is less accurate (±2·0%) than method (*b*) but can yield much useful information.

The chief disadvantage of the continuous monitoring technique, method (*b*), is that only one capillary can be placed in the apparatus, and as each diffusion run may take a week, it takes a long time to obtain a series of results. However, it is more accurate (±0·5%) than method (*a*) and it is also possible to follow changes in the diffusion rate throughout the experiment. This is advantageous in experiments with composite systems, where for example there is a change of interfacial resistance with time.

DIFFUSION OF SODIUM-22 IN ALBUMIN SOLUTIONS AND LECITHIN SOLS

The boundary conditions imposed for the solution of equation (1) require that the concentration of the radioactive ions at the open end of the capillary be zero, and to comply with this it is necessary to have a large volume of inactive solution surrounding the capillary. This is clearly impracticable when using costly materials like lecithin and albumin. The differential diffusion coefficient for albumin is approximately $6{\cdot}7 \times 10^{-7}$ cm^{2}sec^{-1} (Baldwin, Gosting, Williams & Alberty, 1955; Chatterjee, 1964) and the self-diffusion coefficient of ovalbumin in 10% ovalbumin solution is $3{\cdot}32 \times 10^{-7}$ cm^{2}sec^{-1} (Wang, Anfinsen & Polestra, 1954), almost a hundred times slower than that of the sodium ion. Thus it seemed reasonable to assume that the rate of the diffusion of the albumin out of the capillary was negligible in comparison with that of the sodium ion, and that the slowly diffusing albumin would not markedly alter the self-diffusion coefficient of the sodium ion.

To investigate whether or not this assumption was justified, some measurements were made of the self-diffusion coefficients of sodium ions in solutions containing albumin (i.e. albumin in the capillary and in the bath). About 50 ml of inactive solution was used, but because of the impossibility of obtaining the correct flow conditions in such a small volume the solution was not stirred: it has been shown that the effect of stirring is small (Table 1). The results in Table 2 (Nos 1 and 2) are for two solutions; both contained 0·1M sodium chloride and 10% albumin, but with 0·001M calcium chloride added to the second. In both cases the diffusion coefficient agreed within experimental error for inactive solutions containing no albumin (Table 2, Nos 8 and 9) and it was therefore assumed in the present experiments that the absence of albumin in the inactive solution was not important. A similar conclusion was reached for the diffusion of sodium ions in lecithin sols where the micelles probably diffuse one hundred times slower than the sodium ion (Saunders, 1953; Thomas & Saunders, 1959). The molecular weight of micelles treated with ultrasonic irradiation is of the order of 10^6 (Attwood, 1965) and one would expect them to diffuse slowly.

DIFFUSION IN SYSTEMS CONTAINING MACROMOLECULES

The diffusion coefficients of sodium in albumin solutions and lecithin sols each containing 0·1M sodium chloride are lower than the corresponding value in 0·1M sodium chloride alone. This decrease could be due to the increased diffusion path owing to the presence of large molecules (obstruction effect) or it could be due to the adsorption of sodium ions, or a combination of both factors. Similar decreases have been found in other systems: sodium ion diffusion in solutions of sodium dodecylsulphate (Clifford & Pethica, 1964), and in solutions containing ion-exchange resins (Brady & Salley, 1948; Huinzenga & others, 1950; Dux & Steigman, 1958, 1959), and for the diffusion of water in ovalbumin solutions (Wang & others, 1954).

In experiments with ion-exchange resins two types of ions have been considered; bound ions which are those adsorbed on to the molecule,

and the rest which are classed as "free" ions. An estimate can be obtained of the fraction, F, of total ions bound to the macromolecules using the relationship:

$$F = \frac{C_b}{C} = \frac{D - D'}{D - D_m} \quad .. \quad .. \quad .. \quad (18)$$

where C_b = concentration of bound ions, C = total concentration of ions, D_m = diffusion coefficient of macromolecule, D′ = observed diffusion coefficient of ions, D = diffusion coefficient of ions in medium, containing no macromolecules.

Assuming such a relationship is valid for the systems that we are studying, when the fraction of bound sodium ions is plotted against the albumin concentration, F increases with increasing concentration and then appears to tend to a limiting value. This suggests that F is not solely controlled by the albumin concentration.

It has also been assumed that this relationship (18) is valid for lecithin sols since the critical micelle concentration is negligibly small; values of the fraction bound are given in Table 6. More sodium ions appear to be bound in lecithin sols than in albumin solutions at the same concentration.

TABLE 6. FRACTION OF BOUND SODIUM IONS IN VARIOUS LECITHIN SOLS

Experiment No.	Method	Composition of solution		Fraction bound
		in capillary	in bath	
11	a	0·1M NaCl 5% w/w lecithin	0·1M NaCl	0·064
12	b	"	"	0·056
14	a	0·1M NaCl 0·001M $CaCl_2$ 10% w/w lecithin 5% w/w cholesterol	0·1M NaCl 0·001M $CaCl_2$	0·267
15	b	"	"	0·265

A linear relationship has been suggested by Wang between the apparent diffusion coefficient of water and the concentration of ovalbumin in solution. The albumin systems used in the present work behave less simply, except at low concentrations. This is to be expected because the structure of the solutions will be more complicated than Wang's model due to the presence of sodium chloride.

When calcium chloride was added to a 10% albumin solution it caused an increase in the diffusion rate. This is probably caused by a displacement by the calcium ions of sodium ions adsorbed on to the albumin molecules. Thus less sodium would diffuse with the protein molecule.

The addition of cholesterol and of calcium chloride to a 10% lecithin sol resulted in a large reduction in the diffusion coefficient. This could be caused partly by the increase in the size of the micelles due to the incorporated cholesterol and partly by the greater number of micelles due to the increased concentration of lecithin.

SEMI-INFINITE SYSTEMS

Table 3 illustrates the application of equation (3) to systems where active 0·1M sodium chloride is contained in the capillary and 0·1M sodium chloride in the bath (inactive solution); Table 4 shows the result of applying equation (3) to systems in which either lecithin or albumin with 0·1M sodium chloride (active) is contained in the capillary and inactive sodium chloride in the bath. The results in both Tables show good agreement between experimental and calculated activities. The application of the equations described in section 3 to semi-infinite composite systems consisting of lecithin sols layered on top of albumin solutions is depicted in Table 5. By substituting the total length of the albumin layer (the semi-infinite medium II) for x in equation (11), it was found that there was no change of C(x,t) at the closed end of the tube until t was approximately 6 hr. From this it was concluded that the system was semi-infinite up to 6 hr after the start of the experiment; thereafter the system became finite. It appears that there is no resistance to or facilitation of diffusion at the boundary between the albumin and lecithin layers during this time. However other results, not published here, indicate that under certain conditions a resistance to diffusion at the boundary may exist.

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SHORT COMMUNICATION

A method for the evaluation of some oral prolonged-release forms of dexamphetamine in man, using urinary excretion data

A. H. BECKETT AND G. T. TUCKER

ALTHOUGH urinary excretion studies (Campbell, Nelson & Chapman, 1959; Chapman, Shenoy & Campbell, 1959; Shenoy, Chapman & Campbell, 1959) have been used as a technique to evaluate prolonged-release dosage forms of amphetamine, the significance of the results is in doubt because of the use of non-specific assay methods and the failure to control urinary pH. The use of radioactive material has indicated the similarity between a dose of 5 mg amphetamine three times a day and a 15 mg prolonged release preparation (Rosen, Tannenbaum, Ellison, Free & Crosley, 1965).

Since the urinary excretion of amphetamine is pH-dependent (Beckett & Rowland, 1964; Asatoor, Galman, Johnson & Milne, 1965) excretion rates will reflect drug levels in the plasma only when the renal reabsorption of the drug is negligible (i.e. when the urinary pH is $5{\cdot}0 \pm 0{\cdot}5$). Only under such conditions can the patterns of absorption of the drug from conventional and prolonged-release forms be compared in a meaningful fashion. It should be possible to evaluate the results of changing a drug formulation by the study of drug excretion in a few subjects.

This preliminary communication reports the results obtained with various pellet-type preparations, using a specific and sensitive assay for unchanged amphetamine (Beckett & Rowland, 1965a), and using subjects whose urine was rendered acidic.

EXPERIMENTAL

Trial conditions. Male subjects, 22–26 years, were used. An acidic urine was induced and maintained by ammonium chloride. The regimen was 8 g ammonium chloride (0·5 g enteric coated tablets) taken on the day before the trial (2 g every 4 hr), then 2 g at 1 hr before the dose and 1 g every 4 hr thereafter. Breakfast of tea or coffee and toast was taken 1 hr before the dose of dexamphetamine. Urine was collected at 15 or 30 min intervals for the first 3 or 4 hr, then hourly for 16 hr, followed by a 24 hr sample. For the prolonged-release forms further samples were taken at 2 or 4 hr intervals or both.

Dosage forms. (1) "Free Capsule"—consisting of sugar pellets, coated with dexamphetamine sulphate and contained in a gelatin capsule. (2) "Fast Capsule"—as in (1) except that the pellets were further coated with a thin lipid film designed to delay the release of the drug. (3) "Slow Capsule"—as in (2) but with a thicker lipid coating on the pellets. (4)

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"Free Pellets"—as in (1) but without the gelatin capsule. (5) Preparation A—was a product consisting of a mixture of pellets, contained in a gelatin capsule: (20% of the pellets as in (1); 30% as in (2); 10% as in (3) and the remainder with lipid coatings of intermediate thicknesses). Dose: 15 mg dexamphetamine sulphate. (6) Preparation B—was a commercial product consisting of drug pellets, each coated with a material forming a dialysing membrane, contained in a gelatin capsule. In this instance the pellets were claimed to be identical. Dose: 15 mg dexamphetamine sulphate (product also contained 60 mg amylobarbitone).

Regimens. (A) To establish the individual release properties of the pellet groups used in Preparation A, the excretion of dexamphetamine was compared after 15 mg doses in each of the forms (1), (2) and (3). These trials also served to indicate the efficiency with which dexamphetamine is absorbed in different parts of the alimentary tract.

(B) The dexamphetamine pattern of excretion after a single 15 mg dose given in form (1) was compared with those obtained after administration of each of the prolonged-release products A and B.

(C) A divided dose regimen of 5 mg doses in form (1) given at 0, 4 and 8 hr was investigated. The pattern of excretion after this regimen was also compared with those for the prolonged-release products A and B.

(D) The possibility of a dose-effect in the elimination of dexamphetamine was examined by comparing dexamphetamine excretion after a single 5 mg dose in form (1) with its excretion after a single 15 mg dose in the same form.

(E) The effect of the gelatin capsule on the absorption of the dexamphetamine was examined by comparing the excretion of the drug after a 15 mg dose in form (1) with its excretion after the same dose in form (4). A 15 mg dose of dexamphetamine in aqueous solution was also given, as a further control, in one subject.

Each of the above regimens was investigated in three subjects, but only one subject received the solution.

Determination of amphetamine. Amphetamine, in urine, was determined by the gas-liquid chromatographic method described by Beckett & Rowland (1965a).

Calculations. In the post-absorptive phase, under acidic urine conditions, the elimination of dexamphetamine declines exponentially. The biological half-life ($t_{\frac{1}{2}}$) and the amount of drug excreted at infinite time were calculated by standard methods (Beckett & Rowland, 1965b). The percentage of the dose absorbed at various times was calculated using the equation derived by Wagner & Nelson (1964), for urinary excretion data.

RESULTS AND DISCUSSION

The effect produced on the net absorption rate of dexamphetamine, by varying the thickness of the lipid coating on the pellets was reflected in the patterns of excretion obtained with forms (1), (2) and (3). A peak excretion rate of dexamphetamine was established after about 3 hr with form (1), 5–6 hr with form (2), and 11–14 hr with form (3). The results

indicated that dexamphetamine is absorbed throughout a substantial portion of the alimentary tract.

The capsule delays the time for complete absorption of the dexamphetamine from "free" forms by $1\frac{1}{2}$ to 2 hr. The absorption rates calculated in the subjects given a 15 mg dose in solution or "free pellet" forms were similar to those obtained by Beckett & Rowland (1965b) using aqueous solutions of amphetamine.

Fig. 1 shows typical patterns of dexamphetamine excretion obtained with various dosage forms and regimens in subject J.F.T. Similar curves were obtained for the other subjects. Both preparations A and B (see curves IV and V in Fig. 1) eliminated the marked "peaking" effect produced by the single 15 mg "free capsule" form (see curve I, Fig. 1), and the "staircase" effect produced by the 5 mg "free capsule" form given three times a day (see curve II, Fig. 1).

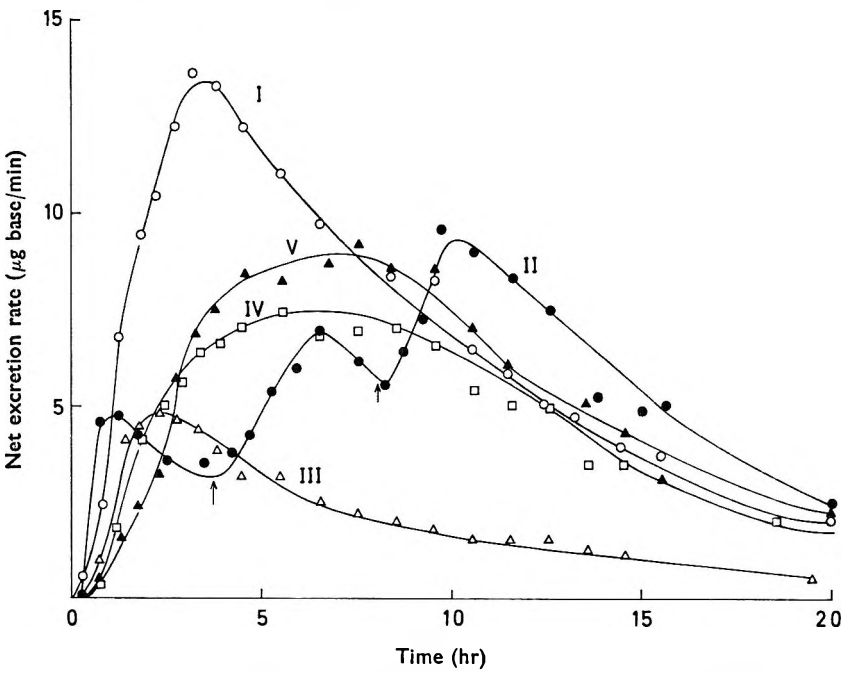

FIG. 1. Urinary excretion of amphetamine after oral administration of dexamphetamine sulphate in different dosage forms and regimens. Subject: J.F.T. Acidic urine control. Curve I —○— 15 mg "Free Capsule". Curve II —●— 3 × 5 mg "Free Capsule" at 4 hr intervals (↑) (first dose given as "Free Pellets"). Curve III —△— 5 mg "Free Capsule". Curve IV —□— 15 mg Prolonged-release Preparation A. Curve V —▲— 15 mg Prolonged-release Preparation B.

A comparison of curves IV and V in Fig. 1 shows that appreciable excretion rate levels of dexamphetamine were established slightly more rapidly with preparation A than with preparation B. This is due to the presence of a portion of the dose, in preparation A in the "free" form. The pattern produced by preparation B showed excellent reproducibility

from subject to subject, whereas that of Preparation A was variable, suggesting a slightly more erratic drug release.

In one subject (G.T.T.), following preparation A, there were further small excretion peaks at 14 and 24 hr after dosage).

Provided that ammonium chloride does not interfere with drug release and absorption, curves IV and V in Fig.1 indicate that preparations A and B were capable of prolonging the absorption of dexamphetamine and of producing reasonably sustained release of the drug for 7 to 8 hr.

In general, no marked inter-or intra-subject variations in the elimination of dexamphetamine were found when a 15 mg "free" dose was given on several occasions. This suggests that it is not necessary to use large numbers of subjects when evaluating dosage forms of the drug by the present method.

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The metabolism and excretion of lignocaine in man

A. H. BECKETT*, R. N. BOYES* AND P. J. APPLETON†

The urinary excretion of lignocaine and monoethylglycinexylidide (MEGX) has been studied in man. The finding that MEGX is a major metabolite of lignocaine in man is discussed in relation to studies in animals reported by other workers. An analogue computer model was constructed to simulate the metabolism and excretion of lignocaine. By varying the model parameters it was possible to obtain good agreement between the calculated and empirical excretion data for both lignocaine and its metabolite MEGX. Because of this good agreement, the computer was used to predict body levels of the compounds after a dose of lignocaine.

DESPITE the extensive clinical use of lignocaine, little is known of its biological fate in man, although extensive studies, both *in vivo* and *in vitro*, have been made in animals. Sung & Truant (1954) reported that in man (two subjects) only 3–11% of an administered dose of lignocaine was excreted unchanged in the urine. More recently, Eriksson & Granberg (1965) found that the renal clearance was affected markedly by changes in urinary pH but not significantly by urine flow rate.

After intravenous administration of lignocaine to dogs, McMahon & Woods (1951a) found no evidence for the splitting of the amide bond but, based on an increase in the excretion of organic sulphate, they suggested ring hydroxylation followed by conjugation with sulphate (1951b). However, Geddes & Douglas (1956) incubated ^{14}C-labelled lignocaine with rat liver slices and suggested that hydrolysis of the amide bond occurred, resulting in diethylaminoacetic acid as the main metabolite (Geddes, 1958). Hollunger (1960a, b) studied the properties of the liver enzyme system responsible for the metabolism of lignocaine in rabbits and found that lignocaine was dealkylated to the monoethyl derivative, which was then rapidly hydrolysed at the amide bond. The secondary amine was much more susceptible to hydrolysis than either the tertiary (lignocaine) or the primary (glycinexylidide) amine, and little or no primary amine was produced as a metabolite of lignocaine.

The present paper correlates previous results obtained in animals with the elimination of lignocaine in man.

Experimental

Materials and Apparatus. Lignocaine hydrochloride. Monoethylglycinexylidide hydrochloride (MEGX). Glycinexylidide hydrochloride. Analar diethyl ether, freshly distilled. Internal marker solution: chlorpheniramine maleate 5·7 μg/ml. 5N Sodium hydroxide. 0·5N Hydrochloric acid. Perkin Elmer Model F 11 Gas Chromatograph. TR-20R Analogue Computer (Electronics Associates Ltd.).

URINE EXCRETION TRIALS

Three male volunteers were given 5 ml intravenous injections of lignocaine hydrochloride, and on a separate occasion a 5 ml injection of MEGX, both equivalent to 50 mg of lignocaine base. The urine was maintained

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at a constant acid pH by the oral administration of ammonium chloride according to the following regimen: 2 g every 4 hr for half a day before the injection, 2 g 2 hr, and 1 g 1 hr before the injection, and 1 g every 4 hr thereafter. Urine was collected hourly for 12 hr after the dose, and one overnight sample was taken the following morning. Lignocaine and MEGX were determined in urine by the method of Beckett, Boyes & Parker (1965). Glycinexylidide, added to urine, was also detected by this procedure.

ANALYSIS OF BLOOD SAMPLES

After each lignocaine injection blood samples were taken at regular intervals for 1 hr (Beckett & others, 1965).

The extraction procedure was improved by the following modification. Blood (2 ml), water (3 ml), carbon tetrachloride (1 ml) and 5N sodium hydroxide (0·5 ml) were placed in a glass-stoppered centrifuge tube. The mixture was shaken (5 min), centrifuged, and as much as possible of the aqueous layer was carefully removed and discarded. To the residual carbon tetrachloride were added 0·5N hydrochloric acid (2 ml) and 1 ml of the internal marker solution. The tube was shaken (5 min), centrifuged, and the aqueous layer transferred to a second centrifuge tube. The carbon tetrachloride layer was further extracted with 0·5N hydrochloric acid (2 ml). The combined acid extracts were made alkaline with 5N sodium hydroxide (1 ml) and the remainder of the extraction and chromatography was carried out using the previously described procedure.

COMPUTER ANALYSIS OF RESULTS

In view of the work of Hollunger (1960a, b) and Geddes (1958) with animals, the model shown in Fig. 1 is proposed as a basis for studying the

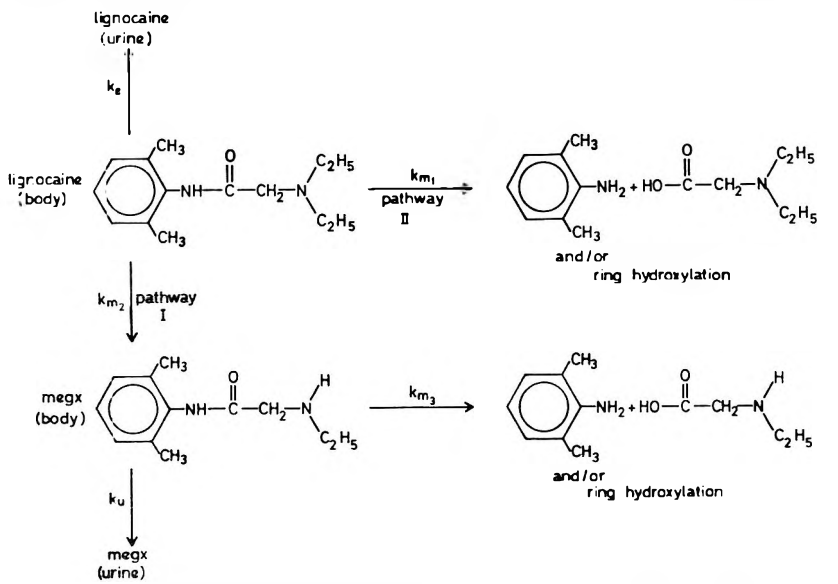

FIG. 1. Proposed pathways for the elimination of lignocaine in man.

elimination of lignocaine in man. Assuming first-order rate processes and a constant volume of distribution, the analogue computer was programmed, as illustrated diagramatically in Fig. 2, to evaluate the various rate processes involved in the model. The various rate constants were systematically altered on the computer until a fit was obtained between the computer output and the experimentally determined excretion values

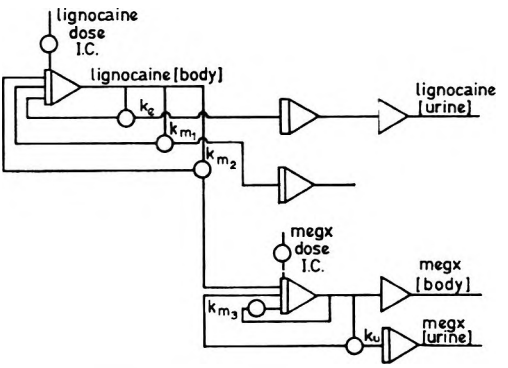

FIG. 2. Analogue computer programme for the elimination of lignocaine in man.

for lignocaine and MEGX for each subject. The MEGX excretion data were analysed by placing the initial dose value directly into the MEGX (body) compartment. When a fit was obtained between the experimental and computer curves, rate constants were determined from the appropriate potentiometer settings.

Results

The rates of excretion of lignocaine and its metabolite, MEGX, after an intravenous dose of lignocaine, alone are shown in Fig. 3. Although glycinexylidide could not be detected in any of the urine samples analysed, the primary aromatic amine (xylidine) was detected gas chromatographically after doses of lignocaine and MEGX. However, due to the very small quantities observed and the instability of this compound in aqueous solution no attempt was made to draw conclusions of a quantitative nature from these results. The 12 hr recoveries of lignocaine and MEGX in urine, after single doses of each drug, are summarised in Table 1. The

TABLE 1. THE 12 HR RECOVERIES OF LIGNOCAINE AND MEGX IN URINE EXPRESSED AS PERCENTAGES OF THE TOTAL DOSE ADMINISTERED

Subject	Dose	% of dose recovered as: Lignocaine	MEGX
P.A.	Lignocaine	7·2	3·5
	MEGX		12·5
D.E.	Lignocaine	4·1	5·0
	MEGX		12·5
J.H.	Lignocaine	6·9	4·0
	MEGX		12·3

blood levels of lignocaine, in all cases, declined rapidly from about 1 μg/ml such that after 45 min the concentration was below the limit of accurate determination by the method used.

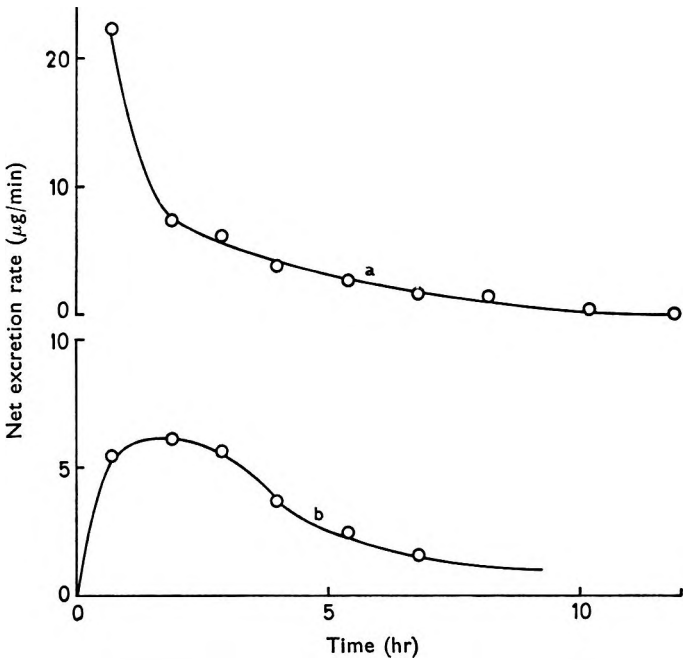

FIG. 3. Rates of urinary excretion of lignocaine (a) and MEGX (b) after an i.v. dose of lignocaine HCl equivalent to 50 mg lignocaine base. Subject P.A.

Agreement between the curves produced by the computer (continuous curves) and the experimental excretion data (points) for lignocaine and MEGX after a single dose of lignocaine is illustrated by curves (a) and (b), respectively, of Fig. 4 (subject P.A.). Similar agreement was also obtained for the other subjects. The good agreement suggested extrapolation of the excretion data to describe the amounts of the compounds in the other compartments of the model. For example, curves (c) and (d) of Fig. 4

TABLE 2. CALCULATED RATE CONSTANTS FOR THE ELIMINATION OF LIGNOCAINE AND MEGX FROM MAN AND THE PERCENTAGE OF THE TOTAL DOSE OF LIGNOCAINE METABOLISED IN EACH PATHWAY

Subject	Dose	k_e •	k_{m1}	k_{m2}	k_{m3}	k_u	Pathway: I	Pathway: II
P.A.	Lignocaine	0·027	0·190	0·161	0·771	0·074	42·8	50·2
	MEGX				0·414	0·060		
D.E.	Lignocaine	0·016	0·200	0·169	0·606	0·071	43·8	52·4
	MEGX				0·465	0·068		
J.H.	Lignocaine	0·030	0·209	0·200	0·780	0·077	45·6	47·6
	MEGX				0·425	0·061		

• Units: hr^{-1}.

represent the changes in the body levels of lignocaine and MEGX respectively with time. These are complementary to the excretion curves (a) and (b) in this figure.

Table 2 shows the rate constants calculated by the computer for each subject. Provided the assumptions inherent in the model are correct, it also shows the amount of lignocaine passing through each pathway of metabolism.

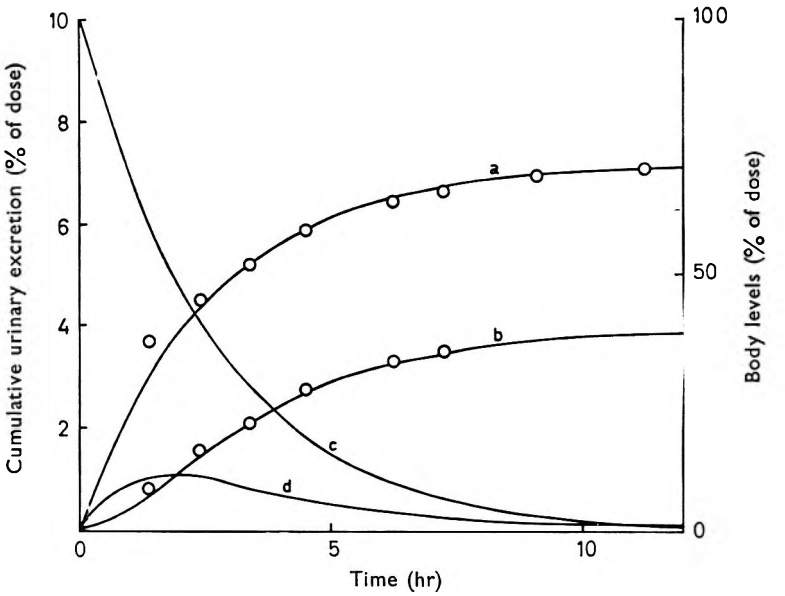

FIG. 4. Agreement between computer calculated and experimental cumulative excretion data for lignocaine (a) and MEGX (b) after a dose of lignocaine. Curves (c) and (d) are calculated body level - time plots for the same compounds. Continuous lines: analogue computer curves; open circles: experimental excretion data. Subject P.A.

Discussion

The use of an analogue computer to develop model systems which can simulate drug distribution is an established procedure (see Garrett, Thomas, Wallach & Alway, 1960; Taylor & Wiegand, 1962).

In the present study, accurate experimental data for the urinary excretion of lignocaine and its metabolite, MEGX, have been obtained under conditions of constant acid urinary pH (see Fig. 3). In the computer simulation each of the rate constants in the model has a distinct influence on the solution of the differential equations involved. With the exception of the ratio of k_{m1} to k_{m2}, approximations of all of the constants can easily be obtained from the experimental data by classical means. The constants presented in Table 2 were those which resulted in the best agreement between the empirical data and that calculated by the computer (see Fig. 4, curves a and b). Thus the proposed model may be used initially as a reasonable approximation of the metabolism and excretion of lignocaine in man and

the calculated results for the other compartments in the model accepted as satisfactory approximations.

The low recovery of unchanged lignocaine in the urine (see Table 1) indicates substantial metabolism of the drug in man. Since 3–5% of the lignocaine dose was excreted as MEGX, and only about 12% of a dose of MEGX is excreted unchanged (see Table 1), it may be concluded that dealkylation is probably a major pathway in the metabolism of lignocaine. As shown in Table 2, the computer indicates approximately 42% of the dose was metabolised by this pathway. Such substantial dealkylation in man is in agreement with Hollunger's (1960a, b) findings in rat and rabbit liver microsomes. The fact that glycinexylidide was not detected in any of the urine samples analysed suggests that if the dealkylation of the secondary amine MEGX occurs in man, it is a slow process.

Table 2 shows that there are differences between the sum of k_u and k_{m3} for MEGX when given as an intravenous dose and when present as a metabolite of lignocaine. Differences in the volumes of distribution of MEGX, when given intravenously and when formed as a metabolite, may explain the changes in the sum of the constants under these separate conditions: similar results have also been obtained for ephedrine and its metabolite norephedrine (Wilkinson, 1966). It has also been suggested (Portmann, McChesney, Stander & Moore, 1966) that there may be a difference in the volume of distribution for hydroxynalidixic acid when given orally and when formed as a metabolite of nalidixic acid.

The very low blood levels of lignocaine 45 min after an intravenous injection indicated a high extravascular concentration of this compound. The body levels of lignocaine and MEGX predicted by the computer (Fig. 3, curves c and d) show that, after 2 hr, 45% of the administered dose of lignocaine remains unchanged in the body and 12% is present as MEGX; the presence of MEGX in the body may contribute to the toxicity of lignocaine on prolonged administration.

Acknowledgements. We are indebted to our colleagues who acted as subjects in this investigation. We thank A. B. Astra, Södertälje, Sweden for supplying samples of monoethylglycinexylidide hydrochloride and glycinexylidide hydrochloride.

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The *in vivo* metabolism of isomeric methoxyoxindoles

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Isomeric methoxyoxindoles are metabolised by rats, guinea-pigs and rabbits to phenolic metabolites by *O*-demethylation and hydroxylation. This has been shown by comparison of the physical characteristics of the metabolites with those of standard synthetic hydroxyoxindoles. Negligible *O*-demethylation of methoxyoxindoles occurs with rat or guinea-pig liver microsome preparations, when compared with those of rabbit, but significant *O*-demethylation occurs with rat liver slices.

A MAJOR route of metabolism of many aromatic alkyl ethers involves cleavage to the corresponding phenols (Axelrod, 1956) by those enzyme systems, located in the microsomes of liver, requiring both reduced nicotinamide adenine dinucleotide phosphate and oxygen.

It has been shown previously (Beckett & Morton, 1966b) that methoxyoxindoles are *O*-demethylated by rabbit liver microsome preparations, but not by rat or guinea-pig liver microsome preparations.

As part of a wider study on the biotransformation of oxindole alkaloids, the dealkylation of isomeric methoxyoxindoles *in vivo* has been examined.

Since conjugated *O*-demethylated metabolites are present in the urine of rats and guinea-pigs after parenteral administration of methoxyoxindoles, further experiments with rat liver homogenates and slices are described.

Experimental

MATERIALS AND METHODS

The preparation of standard synthetic hydroxyoxindoles has been previously described (Beckett & Morton, 1966a).

In vivo experiments. Male Wistar rats weighing 300–350 g, male Flemish rabbits weighing 2·5–3·0 kg and male albino Wistar guinea-pigs weighing 450–500 g were used. The animals received intraperitoneal injections (1·0 ml) of 4-, 5-, 6- or 7-methoxyoxindole (60 mg/kg) in propylene glycol. The faeces-free urine was collected for 96 hr and the metabolites extracted as previously described (Beckett & Morton, 1966a).

In vitro experiments. The animals used were similar to those previously described (Beckett & Morton, 1966b). Some of the rats received intraperitoneal injections of 3,4-benzpyrene (40 mg/kg) 48 hr before being killed.

Tissue preparation. The microsome fraction and microsome plus soluble fractions of liver were prepared as described by Beckett & Morton (1966a). Liver slices, approximately 250 μ in thickness, were prepared by the freehand method using a razor blade, and immediately placed in isotonic phosphate Ringer solution (Dickens & Šimer, 1930) maintained at 37° ($\pm$0·5°).

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Incubation experiments. The incubation experiments using liver homogenates were made in 50 ml Erhlenmeyer flasks, each containing 100 μmoles nicotinamide, 0·8 μmoles NADP, 0·8 μmoles NAD, 20 μmoles glucose-6-phosphate, 60 μmoles magnesium chloride, 0·1M phosphate buffer pH 7·6, 12·0 μmoles substrate and 2·0 ml of liver homogenate in a total volume of 7·0 ml.

Experiments using liver slices were made in 50 ml Erhlenmeyer flasks, each containing 1·0 g (wet weight) of liver slices, 6·0 μmoles of substrate and isotonic phosphate Ringer solution pH 7·4 in a total volume of 10·0 ml. Flasks were shaken (60 min) in air at 37° ($\pm$0·5°). Controls with inactivated tissue, and controls in which the substrate was omitted were also used.

In vitro *demethylation.* The demethylations were made in the same incubation medium to which semicarbazide hydrochloride (70 μmoles) had been added. The resulting incubate was assayed for formaldehyde by the method of Cochin & Axelrod (1959).

Extraction of in vitro *metabolites.* After completion of the incubation period, the liver slices were homogenised in an all-glass Potter-Elvehjem homogeniser, and the protein was precipitated immediately by adding to each flask zinc sulphate solution (20% w/v) (2·0 ml) and a saturated solution of barium hydroxide (2·0 ml). The mixtures were centrifuged (5 min) at 10,000 *g*. Extractions of the supernatant with 2 volumes of n-butanol were made at pH 7·0. The combined extracts were evaporated almost to dryness under reduced pressure, and examined by thin-layer chromatography. The control incubations were extracted and examined in the same way.

Thin-layer chromatography and ultraviolet spectroscopy of the metabolites and reference compounds were as described by Beckett & Morton (1966b).

TABLE 1. URINARY METABOLITES AFTER INTRAPERITONEAL INJECTION OF 4-METHOXYOXINDOLE TO RATS. (Identical results were obtained with the urine extracts of guinea-pig and rabbit)

Metabolite	Rf value*		Chemical colour reactions			
	System A	System B	DQC reagent	Diazotised *p*-nitroaniline reagent	Naphthoresorcinol reagent	Rhodizonic acid/acid $BaCl_2$ reagent†
4-Methoxyoxindole metabolite A	28	38	blue	—	—	+++
Metabolite A after hydrolysis	81	85	blue	yellow	—	—
4-Methoxyoxindole Metabolite B	26	33	magenta	—	—	+++
Metabolite B after hydrolysis	76	79	magenta	brown	—	—
4-Methoxyoxindole Metabolite C	3	9	blue	—	blue	—
Metabolite C after hydrolysis	82	85	blue	yellow	—	—
4-Methoxyoxindole Metabolite D	2	7	magenta	—	blue	—
Metabolite D after hydrolysis	75	78	magenta	brown	—	—
4-Hydroxyoxindole	82	84	blue	yellow	—	—
5-Hydroxyoxindole	75	72	magenta	brown	—	—
6-Hydroxyoxindole	77	76	grey	orange-red	—	—
7-Hydroxyoxindole	85	86	red-brown	brown	—	—

* *Solvent systems:* A. Chloroform-methanol (4:1). B. Chloroform-methanol-acetone (2:1:7). Silica Gel "G" ("E. Merck", Darmstadt). Chromatogram thickness 250 μ.

† Parke, 1960.

Results

IN VIVO

After intraperitoneal administration of the isomeric methoxyoxindoles to rats, the metabolites shown in Tables 1, 2 and 3 were detected in urine. These metabolites were characterised as shown in the Tables. In addition, the ultraviolet absorption spectra of the metabolites were identical with authentic samples. The metabolites of the isomeric methoxyindoles are listed in Table 4.

TABLE 2. URINARY METABOLITES AFTER INTRAPERITONEAL INJECTIONS OF 5- AND 6-METHOXYOXINDOLE TO RATS. (Identical results were obtained with the urine extracts of guinea-pig and rabbit)

Metabolite	Rf value*		Chemical colour reactions			
	System A	System B	DQC reagent	Diazotised *p*-nitro-aniline reagent	Naphtho-resorcinol reagent	Rhodizonic acid/acid $BaCl_2$ reagent
5-Methoxyoxindole Metabolite E	23	37	magenta	—	—	+++
Metabolite E after hydrolysis ..	75	73	magenta	brown	—	—
5-Methoxyoxindole Metabolite F	2	10	magenta	—	blue	—
Metabolite F after hydrolysis ..	76	72	magenta	brown	—	—
6-Methoxyoxindole Metabolite G	26	36	grey	—	—	+++
Metabolite G after hydrolysis ..	78	76	grey	orange-red	—	—
6-Methoxyoxindole Metabolite H	3	10	grey	—	blue	—
Metabolite H after hydrolysis ..	78	76	grey	orange-red	—	—
4-Hydroxyoxindole	82	84	blue	yellow	—	—
5-Hydroxyoxindole	75	72	magenta	brown	—	—
6-Hydroxyoxincole	77	76	grey	orange-red	—	—
7-Hydroxyoxindole	85	86	red-brown	brown	—	—

Examination of the spot area and density of colour on thin-layer chromatograms, showed that approximately equal quantities of the *O*-demethylated and hydroxylated metabolites were formed after administering 4- and 7-methoxyoxindole to rats. (In the absence of authentic

TABLE 3. URINARY METABOLITES AFTER INTRAPERITONEAL INJECTIONS OF 7-METHOXYOXINDOLE TO RATS. (Identical results were obtained with the urine extracts of guinea-pig and rabbit)

Metabolite	Rf value*		Chemical colour reactions			
	System A	System B	DQC reagent	Diazotised *p*-nitro-aniline reagent	Naphtho-resorcinol reagent	Rhodizonic acid/ $BaCl_2$ reagent
7-Methoxyoxindole Metabolite J	27	36	red-brown	—	—	+++
Metabolite J after hydrolysis ..	86	87	red-brown	brown	—	—
7-Methoxyoxindole Metabolite K	21	31	magenta	—	—	+++
Metabolite K after hydrolysis ..	79	81	magenta	brown	—	—
7-Methoxyoxindole Metabolite L	7	11	red-brown	—	slight blue	—
Metabolite L after hydrolysis ..	86	86	red-brown	brown	—	—
7-Methoxyoxindole Metabolite M	4	5	magenta	—	slight blue	—
Metabolite M after hydrolysis ..	80	81	magenta	brown	—	—

samples of 5-hydroxy-4-methoxyoxindole and 5-hydroxy-7-methoxyoxindole, it is assumed that equi-molecular quantities of these compounds give spot sizes and colour intensities similar to those exhibited by 5-hydroxyoxindole.)

TABLE 4. RAT URINARY METABOLITES OF ISOMERIC METHOXYOXINDOLES

Compound administered	Metabolites detected	
	Ethereal sulphate conjugate	B-Glucosiduronic acid conjugate*
4-Methoxyoxindole	4-Hydroxyoxindole 5-Hydroxy-4-methoxyoxindole	4-Hydroxyoxindole 5-Hydroxy-4-methoxyoxindole
5-Methoxyoxindole	5-Hydroxyoxindole	5-Hydroxyoxindole
6-Methoxyoxindole	6-Hydroxyoxindole	6-Hydroxyoxindole
7-Methoxyoxindole	7-Hydroxyoxindole 5-Hydroxy-7-methoxyoxindole	7-Hydroxyoxindole 5-Hydroxy-7-methoxyoxindole

* Minor metabolites.

Similar results were obtained with guinea-pigs, but with rabbits greater amounts of the *O*-demethylated and less of the hydroxylated metabolites were formed.

IN VITRO

Liver microsome preparations. Previous experiments have shown that 4-, 5-, 6- and 7-methoxyoxindoles are significantly *O*-demethylated by rabbit liver microsome preparations, but not by the liver microsome preparations of rats and guinea-pigs (Beckett & Morton, 1966b). Formaldehyde production observed with the rabbit liver microsome preparations may be substantially increased by pretreatment of the rabbits with phenobarbitone.

TABLE 5. FORMALDEHYDE PRODUCTION AFTER INCUBATION OF 4-METHOXYOXINDOLE WITH THE INTRACELLULAR FRACTIONS OF RAT AND RABBIT LIVER

Intracellular fraction	Formaldehyde produced per hr per g of liver (μmoles)*
Rat microsomes in phosphate buffer 7·6	0·03
Rat microsomes plus rat soluble fraction	0·09
Rat soluble fraction	0·0
Rat whole homogenate	0·05
Rat microsomes plus rabbit soluble fraction	0·10
Rabbit microsomes in phosphate buffer 7·6	0·84
Rabbit microsomes plus rabbit soluble fraction	3·75
Rabbit soluble fraction	0·0
Rabbit whole homogenate	2·43
Rabbit microsomes plus rat soluble fraction	1·20

* 12 μmoles of 4-methoxyoxindole were added per g of liver. The data presented represent the average for three animals, all results of which were within ±10% of the recorded values.

Although negligible amounts of formaldehyde were observed in the present experiments, during the incubation of 4-methoxyoxindole with rat liver microsomal or soluble fractions or with the whole liver homogenate (see Table 5), a metabolite formed by hydroxylation in the 5-position of the

aromatic ring of the oxindole nucleus was present in the incubation extracts. The *O*-demethylating activity of the rat microsome preparations was not increased by pretreatment of the rat with phenobarbitone or 3,4-benzpyrene, or by adding excess quantities of NADP or sodium pyrophosphate to the incubation medium (see Table 6).

TABLE 6. FORMALDEHYDE PRODUCTION AFTER INCUBATION OF 4-METHOXYOXINDOLE WITH NORMAL AND PRETREATED RAT AND RABBIT LIVER MICROSOME PREPARATIONS

Liver fraction and additions	Formaldehyde produced per hr per g of liver (μmoles)*
Untreated rat microsomes plus soluble fraction	0·10
Untreated rat microsomes plus soluble fraction plus 2 μmoles NADP	0·12
Phenobartitone pretreated rat microsomes plus soluble fraction	0·12
Phenobarbitone pretreated rat microsomes plus soluble fraction plus 2 μmoles NADP	0·15
Phenobarbitone pretreated rat microsomes plus soluble fraction plus 50 μmoles sodium pyrophosphate	0·12
3,4-Benzpyrene pretreated rat microsomes plus soluble fractions	0·10
3,4-Benzpyrene pretreated rat microsomes plus soluble fraction plus 2 μmoles NADP	0·12
Untreated rabbit microsomes plus soluble fraction	2·30
Untreated rabbit microsomes plus soluble fraction plus 2 μmoles NADP	3·75
Phenobarbitone pretreated rabbit microsomes plus soluble fraction	5·48
Phenobarbitone pretreated rabbit microsomes plus soluble fraction plus 2 μmoles NADP	5·72

* 12 μmoles of 4-methoxyoxindole were added per g of liver. The data presented represent the average for three animals, all results of which were within ±10% of the recorded values.

The formaldehyde production observed during the incubation of 4-methoxyoxindole with rabbit liver microsome preparations was markedly reduced when the rabbit microsomes were incubated in the presence of rat liver soluble fractions, but there was no increase in the *O*-demethylating activity of the rat microsomes when they were incubated in the presence of rabbit liver soluble fractions (see Table 5).

Liver slices. Thin-layer chromatograms of the n-butanol extracts after the incubation of 4-methoxyoxindole with rat liver slices, showed the presence of a metabolite with Rf values in different systems and chemical colour reactions and an ultraviolet spectrum, after elution from the chromatogram, identical to those of an authentic sample of 4-hydroxyoxindole (see Beckett & Morton, 1966b). However, no measurable amounts of formaldehyde were produced during the incubations of 4-methoxyoxindole with the rat liver slices. A second metabolite identical in chemical colour reaction and ultraviolet spectrum with an authentic sample of 5-hydroxyoxindole, but differing in Rf values was also present in the extracts of the incubation medium.

Discussion

The metabolic products formed after intraperitoneal injections of 4-, 5-, 6- and 7-methoxyoxindole to rats, guinea-pigs and rabbits are summarised in Tables 1–3. The corresponding unconjugated metabolites have been reported for the same compounds after incubation with rabbit liver microsome preparations (Beckett & Morton, 1966b).

O-Demethylation of the methoxyoxindoles occurs in rats and also in rat liver slices, but not with rat liver microsome preparations. It is unlikely that the *O*-demethylating enzyme systems were inhibited by the homogenisation of the rat liver since *O*-demethylation of codeine and *p*-nitroanisole has been reported with similarly prepared rat liver microsome preparations (Axelrod, 1955; Netter & Seidel, 1964).

Jacobson & Kaplan (1957a,b) have demonstrated that concentrations of pyrophosphatases which cleave NADP and NADPH are higher in rat liver microsome preparations than in similar rabbit liver microsome preparations, but the negligible *O*-demethylating activity of the rat liver preparations, when compared with that of rabbit liver microsome preparations, cannot be attributed solely to the action of pyrophosphatases because the addition of excess quantities of NADP or sodium pyrosphosphate (an alternative substrate) to the incubation media, did not increase the formaldehyde production from the methoxyoxindoles.

It has been shown that pretreatment of rats with 3,4-benzpyrene increases the *O*-demethylation of methoxyacetanilide and codeine by rat liver microsome preparations (Conney, Miller & Miller, 1956; Henderson & Mazel, 1964). Similar pretreatment of rats with 3,4-benzpyrene and phenobarbitone in this present study did not increase the *O*-demethylation of 4-methoxyoxindole.

Failure to observe formaldehyde production despite *O*-demethylation during the metabolism of 4-methoxyoxindole by rat liver slices, may be due to the further incorporation or metabolism of the formaldehyde within the cells, or the inability of the semicarbazide reagent to penetrate the cell membranes and complex with the formaldehyde.

Acknowledgements. We thank the Science Research Council for a research scholarship to D.M.M. We are indebted to Mr. R. W. Daisley of the Department of Pharmacy, Chelsea College of Science and Technology, for the preparations of the hydroxyoxindoles and methoxyoxindoles.

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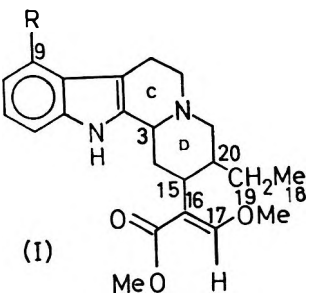
SHORT COMMUNICATION

The *in vitro* metabolism of mitragyna alkaloids of corynantheidine structure

A. H. BECKETT AND D. M. MORTON

CORYNANTHEIDINE has the structure (I) and, apart from *cis-trans* geometry about the C(16)–(17) double bond, four diastereoisomeric pairs of enantiomorphs are possible.



(I)

Alkaloid (I; R = H)	Configuration	C_3H	$C_{20}H$	$C_{15}H$
Corynantheidine	allo	α	α	α
Dihydrocorynantheine	normal	α	β	α
Isocorynantheidine	epiallo	β	α	α
Hirsutine	pseudo	β	β	α

Isocorynantheidine (epiallo) was obtained by C_3H isomerisation of corynantheidine (unpublished observations), and hirsutine was extracted from *M. hirsuta* (Shellard, Beckett, Tantivatana, Phillipson and Lee, 1966); all compounds have $COOMe/OCH_3$ in the *trans* configuration (Lee, Trager & Beckett, 1966).

EXPERIMENTAL

Incubations of these four compounds with rabbit liver microsome preparations were carried out as previously described for the metabolism of oxindole compounds (Beckett & Morton, 1966). Formaldehyde production during the incubations was determined by the method of Cochin & Axelrod, (1959) and the metabolism of the alkaloids by all routes (expressed as a percentage of total alkaloid added to the mixtures) was determined by a method similar to that described by Kato, Chiesara & Vassanelli (1962). Thin-layer chromatography and the determination of partition coefficients were carried out as previously described for oxindole compounds (Beckett & Morton, 1966) and the pK_a values were determined in water by a micro-technique (Jolliffe & Ahmad, unpublished).

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RESULTS AND DISCUSSION

The *in vitro* *O*-demethylation (and metabolism by all routes) of the corynantheidine-type alkaloids was influenced significantly by the stereochemistry of the alkaloids (see Table 1).

TABLE 1. ABSOLUTE CONFIGURATION, pK_a' VALUES AND PARTITION COEFFICIENTS, AND FORMALDEHYDE PRODUCTION AND PERCENTAGE METABOLISM OF CORYNANTHEIDINE-TYPE ALKALOIDS AFTER INCUBATION WITH RABBIT LIVER MICROSOME PREPARATIONS

Alkaloid	Formaldehyde production per hr per gram of liver (μ moles)*	Metabolism by *O*-demethylation (calculated from formaldehyde production) %	Metabolism by all routes†	Partition coefficient Heptane/phosphate buffer pH 7·6	pK_a'‡
Corynantheidine (allo)	0·99	26·0	32·6	332	7·15 ± 0·07
Dihydrocorynantheine (normal)	1·34	35·3	41·5	265	7·47 ± 0·07
Isocorynantheidine (epiallo)	0·58	15·3	23·9	199	7·45 ± 0·04
Hirsutine (pseudo)	0·04	1·1	21·4	249	7·89 ± 0·04

* 3·8μ moles of each alkaloid were added per gram of liver. The data presented represent the average from three animals, all results of which were within ± 10% of the recorded values.

† The metabolism by all routes was derived by difference from the concentration of unmetabolised alkaloid determined by a method similar to that of Kato & others (1962).

‡ The pK_a' values were determined in aqueous solution at 37° (±0·5°).

The results show that the figures for the metabolism by *O*-demethylation, as calculated from formaldehyde produced during the incubation of corynantheidine, isocorynantheidine and dihydrocorynantheine, were not dissimilar from the figures of the metabolism by all routes obtained by determination of unchanged alkaloid after incubation, indicating that *O*-demethylation of these alkaloids was the major metabolic reaction during incubations with rabbit liver microsome preparations. This was confirmed by thin-layer chromatography of n-butanol extracts from incubation mixtures of these alkaloids, because each alkaloid only gave one spot other than the parent alkaloid spot (Table 2). These spots of metabolites gave positive colours with Dragendorff's reagent but negative results with reagents which give colours with phenols. The production of formaldehyde in

TABLE 2. THIN-LAYER CHROMATOGRAPHIC RF VALUES OF CORYNANTHEIDINE-TYPE ALKALOIDS AND THEIR METABOLITES AFTER INCUBATION WITH RABBIT LIVER MICROSOME PREPARATIONS

Alkaloid	Rf values: solvent system*		
	I	II	III
Corynantheidine	0·90	0·74	0·83
Corynantheidine metabolite	0·73	0·51	0·57
O-Desmethylcorynantheidine	0·72	0·51	0·56
Dihydrocorynantheine	0·91	0·76	0·85
Dihydrocorynantheine metabolite	0·78	0·54	0·60
Isocorynantheidine	0·69	0·46	0·67
Isocorynantheidine metabolite	0·50	0·32	0·52
Hirsutine	0·56	0·31	0·51
Hirsutine metabolite	0·35	0·19	0·36

* I. Chloroform-ethanol (9:1). II. Benzene-ethyl acetate (2:3). III. Chloroform-acetone (3:4). Silica gel 'G' (Merck) was used. Chromatogram thickness 250 μ.

these incubations can arise only from oxidative demethylation of the enol-ether on C(17) (see I). Ring hydroxylation is contra-indicated because of the failure of the metabolites to give a colour with phenolic reagents. The *O*-demethylation to produce an OH on C(17) as the major metabolic reaction, under the above conditions, is further confirmed for corynantheidine because the corynantheidine metabolite had properties identical with an authentic sample of *O*-desmethyl corynantheidine (Weisbach & others 1965) on thin-layer chromatograms in three solvent systems.

However, for the pseudo isomer, the 20-fold difference between the percentage metabolism calculated from formaldehyde production and the metabolism by all routes calculated from unchanged alkaloid remaining after incubation, indicated that a different metabolic pathway was involved for this alkaloid. Again one major route was indicated because only one spot other than the parent alkaloid was present on thin-layer chromatograms of n-butanol extracts from the incubation mixtures.

Although the allo, normal and epiallo alkaloids are metabolised by the same route, the formaldehyde production from the epiallo compound indicates that it is only metabolised to about half the extent of the other two alkaloids. There was no direct correlation between heptane-water partition coefficients and pK'_a values (Table 1) and the observed metabolic rates and routes of the alkaloids.

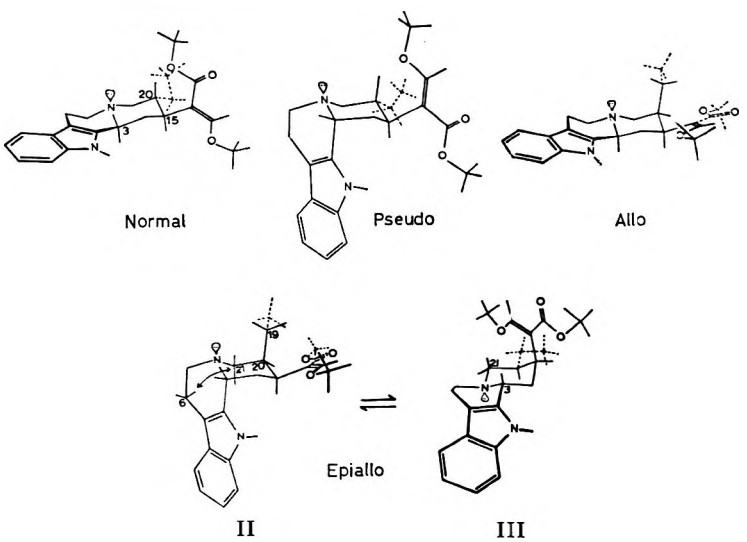

FIG. 1. Preferred conformations of normal, allo and pseudo configurations of corynantheidine-type alkaloids and conformational interchange between the two most important conformations of the epiallo configuration of corynantheidine-type alkaloids

There was, however, some correlation between metabolism and the conformation of these compounds. It has been shown (Lee, Trager & Beckett, 1966) that the normal, pseudo and allo configurations exist at least to the extent of 95% in the conformations shown in Fig. 1, i.e. in the

normal and allo configurations, the indole nucleus is in the general plane of the piperidine ring (D) and in the pseudo configuration it is approximately at right angles to it. Thus, for the planar configurations, *O*-demethylation is the main metabolic route, whereas when the indole nucleus is approximately at right angles to the general plane of the piperidine ring, *O*-demethylation is prevented and another metabolic pathway adopted.

Analysis has shown that in the epiallo configuration in $CDCl_3$, approximately 75% exists in conformation II and 25% in conformation III (Fig. 1). Under aqueous conditions at pH 7·6 the alkaloid will be about 50% ionised. Ionisation and solvation of the protonated nitrogen would increase the steric size of this centre and tend to displace the conformation equilibrium slightly in the direction of III. Thus the epiallo configurations will probably exist in both planar III and non-planar II conformations in roughly equal amounts under the metabolic conditions. The reduced *O*-demethylation of this isomer (epiallo), as compared with those isomers (normal and allo) which exist almost entirely in planar conformation, may thus be explained.

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Solubilisation of preservatives: interaction of benzoic acid with short-chain glycol ethers in water and in aqueous polysorbate 80 solution

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Using a potentiometric titration technique, ethylene glycol mono-ethers (Me, Et, n-Bu, Ph) and diglycol mono-ethers (Me, Et) have been shown to cause elevation of pH in benzoic acid-sodium benzoate solutions. The butyl glycol ether is shown to form micelles; critical micelle concentration values are given and the "loss" of benzoic acid from aqueous solution is considered. In mixtures with polysorbate 80, the glycol ethers increase the uptake of benzoic acid by the micelles.

IN the course of work to study the effect of additives on the distribution of benzoic acid between the aqueous and the micellar phase of a surfactant, the potentiometric method (Donbrow & Rhodes, 1963a,b, 1964, 1965) was applied in the presence of several glycol ethers. These compounds are good solvents as they contain alkyl, ether and alcohol groups in a single molecule; they are somewhat hydrophilic in character, and it was thought that they would have an effect on the hydrophilic lipophilic balance of the surfactant when added to its solution. Members of two series were used which were of the following general formulae: (a) $R{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}OH$ and (b) $R{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}OH$ where R is a short-chain alkyl or an aryl group. Initial work showed that there was a distinct elevation of pH in mixtures of the glycol ethers with polysorbate 80 containing benzoic acid. This paper reports on the phenomenon.

Experimental

EQUIPMENT AND METHODS

Potentiometric titrations. An E.I.L. Vibron Electrometer with a C 33 B-2 unit was used for pH measurement, with GHS 33 glass electrode and calomel electrode (Electronic Instruments Ltd.). The meter was set up in an earthed aluminium box to reduce drift and external electrostatic influences. Linearity of electrode response was checked using three standard buffer solutions covering the pH range studied. The meter was overset 1·0 pH unit during standardisation to enable readings to be made down to pH 2·0. For each titration 5 ml of 0·1M sodium benzoate was used with the appropriate quantity of additives in a total initial volume of 40 ml, and this was titrated with standard 0·1N hydrochloric acid. Titrations were made under nitrogen to exclude carbon dioxide, and the titration cell was thermostatically controlled at 25° (±0·1°).

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Surface tension measurements were made using a Cenco DuNouy tensiometer: solutions were prepared using triple-distilled water.

Refractive index measurements were obtained at 25° using an Abbé Refractometer (Bausch and Lomb Model 3L).

Chemicals. Sodium benzoate, reagent quality; potassium hydrogen phthalate of pH-standard grade (National Chemical Laboratory, certified for use as pH primary standard); 2-ethoxyethanol (BDH); butyl cellosolve (Union Carbide Chemicals); phenoxetol, B.P.C. (Nipa Laboratories); diethylene glycol monoethyl ether (Union Carbide Chemicals) and diethylene glycol monomethyl ether (BDH). All these solvents were purified thus: after standing over calcium carbonate, they were decanted and distilled under reduced pressure, middle cuts of constant b.p. being collected. The boiling points were correct and the free acid contents negligible. Polysorbate 80 (Tween 80, Atlas Chemical Company) was deionised according to Donbrow & Rhodes (1963a).

Solutions were prepared on a v/v basis, using carbon dioxide-free water; they were stored under nitrogen.

Results and discussion

Titration of the sodium benzoate with hydrochloric acid in the presence of 10% polysorbate 80 showed that the addition of 10% of various glycol monoethers produced a significant elevation of pH throughout the titration (Fig. 1 and Table 2). The pH displacements were not caused

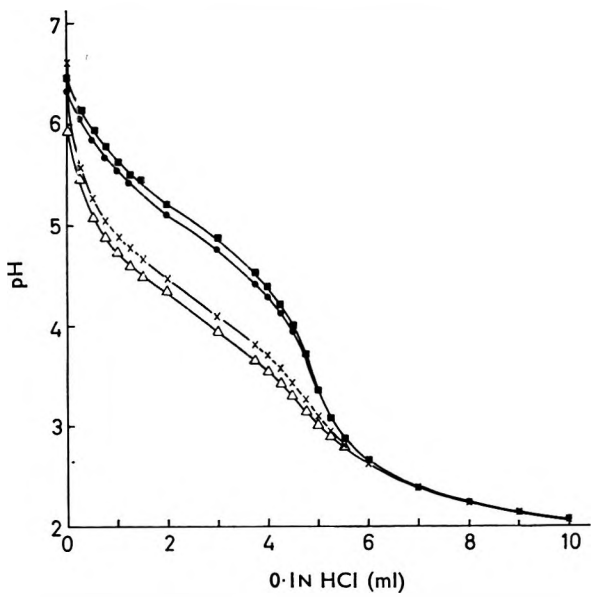

FIG. 1. Titration of sodium benzoate with 0·1N hydrochloric acid in the presence of diethylene glycol monoethyl ether and polysorbate 80. ■, Sodium benzoate + 10% polysorbate + 10% diethylene glycol monoethyl ether. ●, Sodium benzoate + 10% polysorbate 80. ×, Sodium benzoate + 10% diethylene glycol monoethyl ether. △, Sodium benzoate solution.

by basic impurities, as purified materials were used and blanks were run (see Fig. 2), the end-points being identical in all instances.

In the absence of polysorbate, all the monoglycol and diglycol ether-sodium benzoate titration curves were also elevated above the sodium benzoate blank. Up to the level of 10% glycol ether elevations were small but at 20% they were disproportionately large in most instances,

TABLE 1. PH ELEVATIONS OF SODIUM BENZOATE* IN THE PRESENCE OF GLYCOL MONO ETHERS AT 50% NEUTRALISATION. (Simple aqueous solutions—no polysorbate 80.)

	Diglycol ethers		Monoglycol ethers		
% Solvent	Methyl	Ethyl	Ethyl	Butyl	Phenyl
2	0·025	0·025	0·005	0·012	0·052
5	0·075	0·112	0·075	0·125	—
10	0·150	0·162	0·137	0·300	—
20	0·350	0·345	0·375	0·940	—

* pH = 4·125 at half neutralisation point of blank.
ΔpH = pH in glycol ether - pH in blank.
Total benzoate, as salt and free acid, 0·01177M.

the butyl monoether showing the greatest effects (see Fig. 2). Because of solubility limitations, only 2% of the phenyl glycol ether could be titrated. The pH displacements increase in the order—ethyl, butyl, phenyl, though the pH elevations are relatively small at 2% (Table 1). Where no surfactant is present the ethers thus appear to have the general properties of elevating the pH in a similar manner to the surfactant.

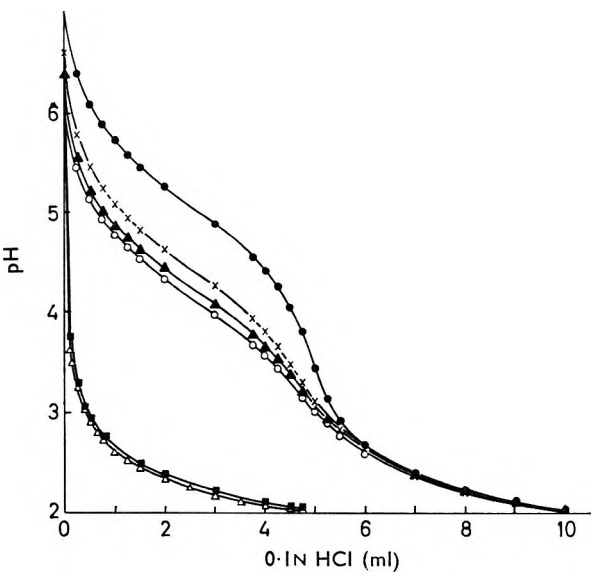

FIG. 2. Titration of sodium benzoate with 0·1N hydrochloric acid in the presence of ethylene glycol monobutyl ether. ●, Sodium benzoate + 20% ethylene glycol ether. ×, Sodium benzoate + 10% ethylene glycol ether. ▲, Sodium benzoate + 5% ethylene glycol ether. ○, Sodium benzoate + 2% ethylene glycol ether. ■, Ethylene glycol ether 10% in water. △, Water blank.

There are several factors operating in solution which might be responsible for an apparent change of pH being observed when a hydroxy ether is added to water or a surfactant solution. Dielectric constant changes in the medium may alter several properties. These are (a) the solubility of a solute and its distribution between two phases, (b) the ionic activity coefficients (so affecting the dissociation equilibrium of benzoic acid), (c) the dissociation constant and the activity of benzoic acid, and (d) the response of the electrode system to a given hydrogen ion activity. On the other hand, the glycol ethers may form micelle-like aggregations, capable of removing the benzoic acid from the aqueous environment or, alternatively, the addition of benzoic acid may assist in the formation and stabilisation of micelles.

TABLE 2. EFFECT OF GLYCOL ETHERS ON THE pH OF SODIUM BENZOATE-POLYSORBATE 80 TITRATION CURVES

	Polysorbate-glycol ether mixture		Glycol ether alone
	pH	ΔpH*	ΔpH†
10% Ethylene glycol ethyl ether + polysorbate 80	4·804	0·049	0·140
10% Ethylene glycol butyl ether + polysorbate 80	4·914	0·159	0·314
10% Diethylene glycol ethyl ether + polysorbate 80	4·860	0·105	0·160
Polysorbate 80	4·755	—	

ΔpH* = pH in (glycol ether + polysorbate 80)-pH in polysorbate 80.
ΔpH† = pH in glycol ether-pH in water.
pH and ΔpH values measured at 60% neutralisation of the sodium benzoate solution (0·01163M total benzoate).

The titration curves in Fig. 2 offer some evidence as to which of these mechanisms might operate. Beyond the end point, the benzoic acid ionisation will be greatly suppressed by excess hydrochloric acid, the concentration of which is identical in each of the solutions. Were the activity coefficient of the hydrochloric acid or the response of the electrode altered, the pH values of the hydrochloric acid at any given burette reading would show a progressive shift with glycol ether concentration. Allowing for scatter, such shifts are not observed in any of the glycols. The results do not favour an explanation in terms of activity coefficient changes or electrode effects.

The disproportionate pH shift observed between 10 and 20% glycol ether is compatible with micelle formation between these concentrations. In contrast, surfactants of low critical micelle concentration (CMC) show a large pH shift at low concentrations, while further shifts decrease with increasing surfactant concentration (Donbrow & Rhodes, 1963a). Surface tension measurements of an aqueous solution of ethylene glycol monobutyl ether gave a curve typical of a surfactant, the sharp break characteristic of the CMC occurring at 11% v/v (Fig. 3). A refractive index plot, showing a break at 12% v/v (Fig. 4), confirmed that the butyl glycol ether forms a micelle-like aggregation (CMC 11–12% v/v, 0·88–0·98M). Elworthy & Florence (1964) obtained similar values (about 0·8M) for C_4n_6 compounds (n = number of oxyethylene groups). The

small difference is not surprising since Mulley & Metcalf (1962) observed that the CMC was little affected by an increase in n value from 3 to 6. However, no values have hitherto been reported for C_4 compounds containing fewer than n_6, or for compounds containing less than n_2 with a carbon chain below $C_{(8)}$ (see Sirianni & Gingras, 1961; Mulley & Metcalf, 1962; Corkill, Goodman & Harrold, 1964). Of the other glycol ethers listed in Table 1, none gave sharp inflexions in the surface tension curves nor showed breaks of slope in the refractive index curves up to 40% v/v.

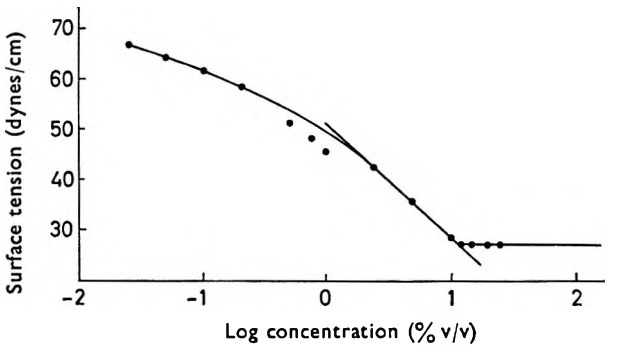

FIG. 3. Surface tension of aqueous solutions of ethylene glycol monobutyl ether at 25° C. Corrected by method of Zuidema & Waters (1941).

The pH shifts reported in Table 1 suggest that some molecular aggregation may also occur in other compounds, the tendency being greatest in the phenyl compound. It is difficult to distinguish between pH effects due to aggregation and to pK_a changes for compounds which do not give a sharp CMC. Parallels cannot be drawn with other solvents of equivalent dielectic constant because of the specific structural factors involved (e.g. see Gutbezahl & Grunwald, 1953). Since the aggregation numbers of these short-chain materials are probably quite small, it

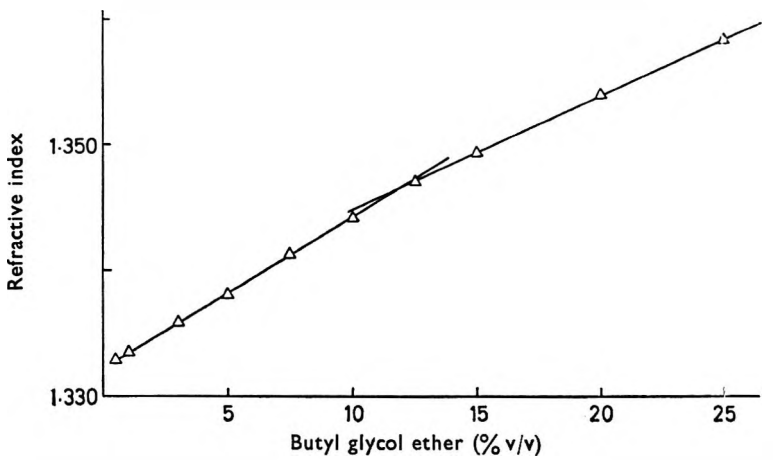

FIG. 4. Refractive index/concentration curve of ethylene glycol monobutyl ether at 25° C.

would be expected from the mass action approach that the transition from monomer to aggregated state would be less sharp than with typical surfactants (Murray & Hartley, 1935); the monomer concentration would therefore continue to increase after micellisation had begun (Elworthy & Macfarlane, 1965). For such compounds, it would be difficult to designate a specific concentration as the CMC, and one would expect properties associated with micellisation, such as solubilisation, to be shown over a wide concentration range. The surface tension curves of the glycol monoethyl ether and the diglycol monomethyl and monoethyl ethers lend some support to this argument; after an initial sharp fall, they continue to decline up to high concentrations. The mechanism of benzoic acid interaction might range from mutual lipophile attraction with orientated solvent molecules, assisted by squeezing-out effects of the water, at one extreme, to complete co-micellisation (Valko & Epstein, 1957) at the other.

The apparent loss of (% free) benzoic acid from the aqueous phase equivalent to such pH shifts is as follows: 86·7, 48·1, 20·5, 12·9 for pH shifts of 0·940, 0·350, 0·162, 0·125 respectively (see Donbrow & Rhodes, 1963b, 1964). As with long-chain surfactants (Wedderburn, 1964), the loss is very large above the CMC of the butyl ether. If the pH changes at lower concentrations are at least partially due to a reduction in benzoic acid activity, it is evident that some care must be exercised in formulating it in preparations containing other amphiphilic substances.

In the polysorbate 80-glycol ether mixtures, the magnitude of the pH shift is similar to that in the polysorbate-free systems (Fig. 1 and Table 2). Most probably the glycol ethers are incorporated in the polysorbate micelles, thereby changing the solubilising power of the surfactant and hence the distribution of the benzoic acid.

Acknowledgements. Thanks are due to Atlas Chemical Industries, Delaware, U.S.A., for a gift of the polysorbate 80, and to Union Carbide Corporation for a gift of glycol ethers used in this study.

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The evaluation of a molecular sieve technique to determine the interaction between a preservative and a surfactant

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The interaction between methyl *p*-hydroxybenzoate and polysorbate 80 has been determined using a molecular sieve technique. At 25° and at surfactant concentrations of 2·86 and 1·43% respectively, the ratios of total to free preservative were 2·9 and 1·9. These results are comparable with published data.

THE use of non-ionic surfactants in pharmaceutical and cosmetic formulations has increased the problem of preserving such preparations against microbial attack. Wedderburn (1964) has reviewed the subject and emphasised the significance of the interaction which can occur between the non-ionic surfactant and the preservative. Although there may be no obvious incompatibility, the thermodynamic activity of the preservative, which for dilute solutions can be equated with its concentration in true aqueous solution, may be reduced. Such a reduction in effective concentration can be studied by microbiological methods and also by physico-chemical techniques (for references see Mitchell & Brown, 1966). The results obtained by physical methods compare well with those obtained microbiologically (Pisano & Kostenbauder, 1959).

In recent years, size separation with molecular sieves has become widely used and we have used this phenomenon to study the degree of binding between a surfactant and a preservative. To evaluate the technique we re-examined the interaction between methyl *p*-hydroxybenzoate and polysorbate 80 and compared the results obtained with those available in the literature and also with the degree of interaction as revealed by a solubility study. Determination of the degree of interaction by molecular sieving with a gel involved three stages: measurement of the external volume of the gel, evaluation of the extent of adsorption of the methyl *p*-hydroxybenzoate on the gel matrix, and determination of the amount of interaction of the preservative with polysorbate 80. An experiment was also made to demonstrate that polysorbate 80 did not penetrate the gel.

Experimental

The material used was Sephadex G-25 fine grade (Pharmacia) which has a nominal water regain of 2·5 g water per g of dry gel. To prepare the swollen gel, 4 g of the dry gel was added to water (15 ml) and the gel was allowed to swell (2 hr). The relevant solutions were then added plus any additional water so that the total volume of liquid added to the gel was always 25 ml. The system was then equilibrated by shaking

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(1 hr) at 25° ± 0·1° (this was shown to be sufficient in preliminary experiments).

Binding of methyl p-*hydroxybenzoate to polysorbate* 80 *in the presence of Sephadex.* Aqueous solutions (5 ml) of known but varying concentrations of methyl *p*-hydroxybenzoate (Nipa) and 5 ml of the appropriate polysorbate (Tween) 80 (Honeywill-Atlas) solution were added to the swollen gel. After equilibration, the systems were allowed to stand at 25° for about 5 min to allow the gel to settle. About 4 ml of the supernatant liquid was pipetted into a tube and centrifuged to deposit any small particles of gel (taking supernatant liquid only has the advantage that it is not necessary to centrifuge at a controlled temperature). The supernatant liquid in the centrifuge tube was then analysed for methyl *p*-hydroxybenzoate by measuring the absorbance at 256 mμ. A correction for the absorbance due to the polysorbate 80 was obtained from a calibration graph.

The adsorption of methyl *p*-hydroxybenzoate to Sephadex was measured as above with the polysorbate 80 solution replaced by water (5 ml).

Determination of the external volume was made using the method described above but with a solution of Blue Dextran (Pharmacia) of average molecular weight 2,000,000 instead of methyl *p*-hydroxybenzoate. The concentration of this in the supernatant was found by measuring the absorbance at 620 mμ.

The degree of binding of methyl *p*-hydroxybenzoate to polysorbate 80 was also assessed by the solubility method of Patel & Kostenbauder (1958) at a temperature of 25° ± 0·1°.

The amount of polysorbate 80 in the Sephadex internal phase was found by equilibrating the swollen gel with solutions of known but varying concentrations of polysorbate 80. After 1 hr, the whole of the external phase was removed by filtering under gentle vacuum. The gel was then well washed with water to remove any surfactant and the washings analysed for polysorbate 80 content by the second method of Stevenson (1954).

Results and discussion

The grade of Sephadex used has a nominal water uptake of 2·5 ± 0·2 g per g of dry gel. This enables the volume of the external phase to be calculated as 14·2 to 15·8 ml. However, as the answer for the degree of interaction between the preservative and the surfactant is dependent on this value it has been determined with greater precision. The mean of nine determinations using Blue Dextran was 15·8 ml.

Gelotte (1960) showed that most aromatic compounds were adsorbed, reversibly, on Sephadex and that a linear adsorption isotherm was obtained. We therefore anticipated that methyl *p*-hydroxybenzoate would also be adsorbed and Fig. 1 is a plot of the concentration of methyl *p*-hydroxybenzoate in the external phase against the weight of the compound associated with the gel. This latter is the sum of that which is in solution in the internal phase and the quantity actually adsorbed on the gel matrix. It is neither necessary to distinguish between these, nor

necessary to know the volume of the internal phase since the quantity of methyl *p*-hydroxybenzoate adsorbed is dependent only on its concentration in the internal phase. This latter must be equal to the external concentration and thus the quantity of methyl *p*-hydroxybenzoate associated with the gel is dependent on the concentration of the compound in the external phase.

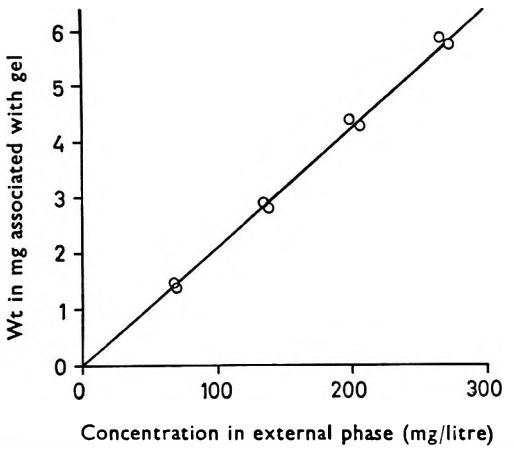

FIG. 1. Relationship between the concentration in the external phase and the weight associated with the gel (adsorbed and in solution) of methyl *p*-hydroxybenzoate at 25°.

In the presence of polysorbate 80, determination of the concentration of methyl *p*-hydroxybenzoate free and bound in the external phase enables the quantity of preservative associated with the gel to be determined, as the total amount of preservative added to the system is known. Fig. 1 gives the concentration of free methyl *p*-hydroxybenzoate in the external phase and thus the ratio of total preservative to free preservative can be calculated. These ratios for the two polysorbate concentrations used are in Table 1 and are plotted in Fig. 2. The results for the solubility studies are given in Fig. 3. Here, the concentration of free preservative is taken as its maximum water solubility under the conditions used.

TABLE 1. BINDING OF METHYL *p*-HYDROXYBENZOATE TO POLYSORBATE 80

Total methyl *p*-hydroxybenzoate/Free methyl *p*-hydroxybenzoate

Method	Molecular Sieve[1]	Solubility	Solubility[3]	Solubility[4]	Dialysis[3,4]
Temperature	25°	25°	27°	27°	30°
Polysorbate 80 conc. 2·85 %	2·86; 2·89 2·88; 2·96	3·10	2·8	2·8	3·0
1·43 %	1·94; 2·14 1·93; 1·97	2·05	1·9	1·8	2·0

1. Concentration range of free preservative was 75 to 190 mg/litre.
2. Concentration range of free preservative was 300 to 630 mg/litre.
3. Blaug & Ansan (1960).
4. Patel & Kostenbauder (1958).

All the results obtained are given in Table 1 and are compared with those reported by previous workers. Apart from one slightly high value for each polysorbate concentration, the values given by the molecular sieve technique agree well with each other and with those obtained with other techniques by earlier workers.

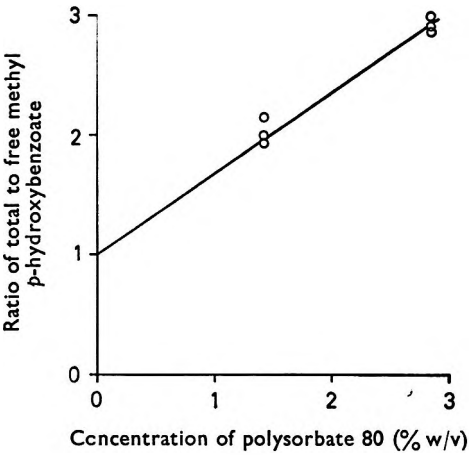

FIG. 2. A plot showing the ratio of total to free methyl *p*-hydroxybenzoate, at 25°, in aqueous solutions containing varying concentrations of polysorbate 80 as determined by a molecular sieve technique.

Although monomeric polysorbate 80 with a molecular weight of about 1300 can penetrate the gel, we considered it unlikely that much would do so in the time used for the experiment (1 hr). Experimental work

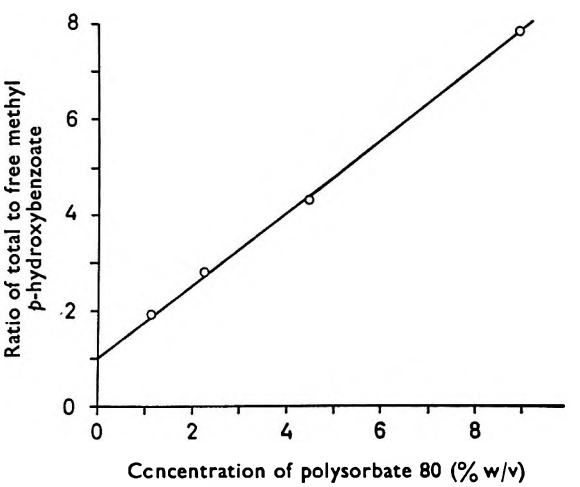

FIG. 3. A plot showing the ratio of total to free methyl *p*-hydroxybenzoate, at 25°, in aqueous solutions containing varying concentrations of polysorbate 80, as determined by a solubility method.

confirmed that for the highest surfactant concentration in the external phase (5·0%), the concentration in the gel was only 0·009%. It is probably less than this because it is not easy to achieve complete separation of the external phase.

Compared with conventional equilibrium dialysis, the use of a gel instead of a semi-permeable membrane has the advantage of rapid equilibration and ready availability of suitable materials of various pore sizes. Errors due to leaching of constituents from the membrane are avoided. Its main disadvantage is that the concentration of preservative is determined in one phase only. Consequently, any error in this determination gives an error in the quantity associated with the gel. Such errors are minimised if the volume of the external phase is chosen so that the quantity of compound in this phase is approximately equal to the quantity associated with the gel.

The method is obviously not restricted to studies of the interaction of preservatives with surfactants. In addition it could probably be used to study drug-protein binding. For this latter, it would seem to offer advantages over the column techniques used with Sephadex by Hardy & Mansford (1962) and Barlow, Firemark & Roth (1962).

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The use of the Coulter Counter to detect the inactivation of preservatives by a non-ionic surface-active agent

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Bacterial and mould spores undergo a germination swelling process which can be accurately measured using a Coulter Counter. The efficacy of preservatives can be assessed by their action in suppressing germination swelling of spores. The antagonism between polysorbate 80 and several preservatives has been demonstrated by measuring swelling of spores in the presence of preservatives and preservative-surfactant mixtures.

AN important factor in the preservation of pharmaceutical and cosmetic two phase systems is the antagonism between non-ionic agents and many preservatives (Wedderburn, 1958, 1964). Physicochemical methods of measuring the interaction between preservatives and non-ionic surfactants have been reviewed (Mitchell & Brown, 1966). These methods include solubility and partition studies, equilibrium dialysis, pH measurements and potentiometric titration. An alternative procedure is to measure the antimicrobial efficacy of preservatives in various combinations with surfactants or in final formulations (Bolle & Mirimanoff, 1950; Barr & Tice, 1957). Although such tests are time-consuming they have the advantage that they use viable micro-organisms and the preservative is assessed under conditions of use. In a modified test of this type Judis (1962) examined the protection given by polysorbate 80 to *Eschericha coli* against chloroxylenol using the release of radioactive material from ^{14}C-labelled cells as an index of cell damage.

The swelling of mould spores during a period of some 5 hr before germ tube formation can be measured rapidly and accurately either by microscopic or by electronic methods. The use of these techniques to evaluate antifungal agents by their effect in suppressing spore swelling has been described by Barnes & Parker (1966). Bacterial spores undergo a similar swelling process which is prevented by antimicrobial agents (Gould, 1964). Measurements of spore swelling should provide a method of rapidly assessing inhibition of preservative action.

Experimental

ORGANISMS

Trichoderma species, IMI 110150. Spore suspensions were prepared from 21 day cultures by the method of Gerrard, Harkiss & Bullock (1960). *Bacillus subtilis* NCTC 3610. Spore suspensions were prepared as described by Gerrard, Parker & Porter (1961).

SIZE ANALYSIS OF DORMANT SPORES

Trichoderma. A suspension of spores in normal saline was submitted to size analysis in a Coulter Counter Model B (with Model J plotter) using a 50 μ orifice tube.

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Instrument settings: Gain trim = 48, Amplification Setting (AS) = 1·0 and Aperture Current (ACS) = 0·5. The average spore volume was 14·8 μ^3. *B. subtilis* spores were analysed in the same manner using a 30 μ orifice tube. Instrument settings: Gain trim = 100, AS = 0·25 and ACS = 0·707. The average spore volume was 0·63 μ^3.

PRESERVATIVE SOLUTIONS

Solutions containing (% w/v) (a) methyl hydroxybenzoate (0·04) and propyl hydroxybenzoate (0·02), (b) chlorocresol (0·34), (c) chlorocresol (0·1), (d) phenylmercuric nitrate (0·004), (e) cetrimide (0·04) and (f) Nipastat (0·1) were prepared in sterile water. All chemicals were of B.P. quality. Nipastat is a mixture of methyl, ethyl, propyl and butyl esters of *p*-hydroxybenzoic acid (Nipa Laboratories Ltd.).

SIZE ANALYSIS OF MOULD SPORES IN PRESERVATIVE SYSTEMS

Malt broth, 4% malt extract B.P.C. in glass distilled water pH5 (10 ml) was mixed with preservative solution (a) (10 ml), inoculated with a suspension (1 ml) of *Trichoderma* spores (approximately 60×10^6) and incubated at 25° for 5 hr. Samples (5 ml) taken from the preservative system and diluted to 50 ml with filtered normal saline were submitted to size analysis as described for dormant *Trichoderma* spores.

A control of malt broth (10 ml) and sterile water (10 ml) similarly inoculated and incubated was also submitted to size analysis. Similar experiments were conducted using the preservative solutions (b)–(f). The results are given in Table 1.

TABLE 1. THE EFFECTS OF PRESERVATIVES, ALONE AND WITH 2% POLYSORBATE 80, ON THE SIZE INCREASE DURING GERMINATION OF *Trichoderma* AND *B. subtilis* SPORES

Preservative	Percentage of swelling of untreated *Trichoderma* spores		Percentage of germination swelling of untreated *B. subtilis* spores	
	Without polysorbate 80	With polysorbate 80	Without polysorbate 80	With polysorbate 80
Nil	100·0	100·0	100·0	100·0
PMN 0·002%	0·0	0·0	0·0	0·0
Chlorocresol 0·17%	0·0	0·0	0·0	0·0
Chlorocresol 0·05%	8·4	75·0	38·4	84·7
Parabens 0·03%	13·4	100·0	12·2	63·0
Nipastat 0·05%	0·0	60·0	13·5	21·0
Cetrimide 0·02%	0·0 (spore disintegration)	66·7	13·5	43·1

SIZE ANALYSIS OF MOULD SPORES IN PRESERVATIVE-POLYSORBATE-80 SYSTEMS

Samples of malt broth (10 ml) each containing 4% w/v polysorbate (Tween) 80 (Honeywill-Atlas Ltd.) were mixed separately with preservative solutions (a)–(f) respectively (10 ml) inoculated with *Trichoderma* spores, incubated at 25° for 5 hr and the suspension submitted to size analysis as described. Malt broth (10 ml) containing 4% w/v polysorbate

80 diluted with sterile water (10 ml) was inoculated, incubated and sampled as a control (Table 1).

SIZE ANALYSIS OF BACTERIAL SPORES IN PRESERVATIVE SYSTEMS

Samples of double strength nutrient broth, Oxoid granules CM 67, pH 7·5 (10 ml) were mixed separately with preservative solutions (a)–(f) respectively (10 ml), inoculated with a suspension (1 ml) of *B. subtilis* spores (approx. 80×10^6) and incubated at 32° for 4 hr. Samples (5 ml) were taken from each preservative system, diluted to 50 ml with filtered normal saline solution and submitted to analysis as described for dormant spores of *B. subtilis*.

A control consisting of equal volumes (10 ml) of double strength nutrient broth and sterile water was similarly inoculated and after incubation submitted to size analysis. The results are given in Table 1.

SIZE ANALYSIS OF BACTERIAL SPORES IN PRESERVATIVE-POLYSORBATE 80 SYSTEMS

Samples of double strength nutrient broth (10 ml) each containing 4% w/v polysorbate 80 were mixed with preservative solutions (a)–(f) respectively (10 ml), inoculated with *B. subtilis* spores and incubated at 32° for 4 hr; samples were size analysed as described. Double strength nutrient broth (10 ml) containing 4% w/v polysorbate 80 diluted with sterile water (10 ml) was inoculated, incubated and sampled as a control (Table 1).

Results and discussion

The average volume of dormant mould spores (14·8 μ^3) increases to 22·5 μ^3 within 4 hr and to 24·5 μ^3 after 5 hr incubation. The germination swelling of the bacterial spores represents an increase in volume from 0·63 μ^3 to 0·86 μ^3 increasing to 2·07 μ^3 on emergence (Fig. 1). At the concentrations in contact with the spores (viz. chlorocresol 0·05 and 0·17, combined hydroxybenzoate esters 0·03, Nipastat 0·05, phenylmercuric nitrate 0·002 and cetrimide 0·02% w/v) the preservatives all suppress the swelling of mould and bacterial spores to some extent. In the presence of chlorocresol (0·05%) and the hydroxybenzoate ester mixture (0·03%) *Trichoderma* spores show an increase in size of less than 15% of that shown by untreated spores. The other preservatives prevent swelling completely and the shape of the analysis plot for cetrimide and mould spores suggests their disintegration. Spores of *B. subtilis* have greater resistance to the preservatives and only phenylmercuric nitrate and chlorocresol at the higher concentration (0·17%) completely suppress swelling. Chlorocresol (0·05%) allowed a swelling of some 38%.

Polysorbate 80 alone (2%) in the nutrient media does not prevent swelling of the spores nor does it inhibit the effect of phenylmercuric nitrate or chlorocresol (0·17%) in suppressing spore swelling (Table 1). The surfactant interferes however with the action of chlorocresol (0·05%), hydroxybenzoate esters, Nipastat and cetrimide. With polysorbate 80 in these preservative systems both mould and bacterial spores swell.

The validity of our experimental approach is confirmed by the demonstration of mutual antagonism known to exist between polysorbate 80 and antimicrobial agents such as chlorocresol, the hydroxybenzoates and cetrimide. These are compounds known to interact with the polyoxyethylenes. The interaction between polysorbate 80 and chlorocresol is of interest in that at a concentration of 0·05% the phenolic compound is inactivated whereas at 0·17% it retains activity.

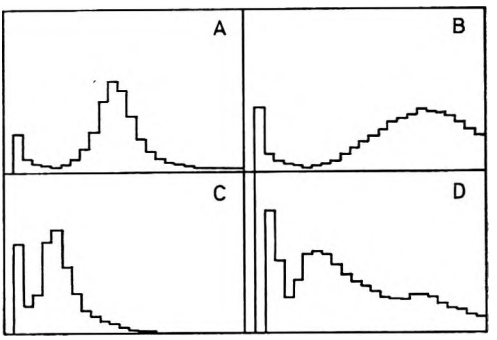

FIG. 1. The increase in size of *Trichoderma* and *B. subtilis* spores during germination, measured with a Model B Coulter Counter and Model J plotter. A. *Trichoderma* spores, peak size 14·81 μ^3. B. *Trichoderma* spores after 5 hr incubation, peak size 24·5 μ^3. C. *B. subtilis* spores, peak size 0·63 μ^3. D. *B subtilis* spores after 4 hr incubation, peaks at 0·86 μ^3 and 2·07 μ^3.

Preliminary investigation of aqueous mixtures of chlorocresol (absorption maximum 280 mμ) and polysorbate 80 (absorption maximum 234 mμ) shows that between 220 mμ and 300 mμ their absorption curves are the simple summation of the curves of their components. The precise nature of the interaction between the surfactant and chlorocresol is being studied.

It has been reported that phenylmercuric nitrate is not inactivated by polysorbate 80 (2%) (Wedderburn, 1958) and in agreement we found a 2% concentration did not interfere with the efficiency of the preservative (0·002%). As anticipated we found that the level of surfactant used (2%) inactivated the hydroxybenzoate ester mixture, the proprietary mixture (Nipastat) and the cetrimide at the concentrations used.

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The absorption by *Escherichia coli* of phenols and their bactericidal activity

H. S. BEAN AND A. DAS

The uptake of several phenols by *E. coli* may be represented by absorption isotherms. The uptake pattern for dilute solutions suggests partitioning between the cells and aqueous solution but at higher concentrations a change in the uptake pattern suggests protein precipitation. An inflexion in the absorption isotherms corresponds with concentrations producing high levels of activity and can be used to forecast concentrations needed to produce rapid rates of kill.

EARLY studies of the kinetics of disinfection established a correlation between the concentration of a bactericide in aqueous solution and the rate of disinfection of a test culture (Chick, 1908) which suggested that the bactericidal reaction is either a true monomolecular reaction or that it simulates a monomolecular reaction. It is known (Nernst, 1917) that reactions in heterogeneous systems are regulated by diffusion and that the kinetics of such reactions always resemble those of a monomolecular reaction. Thus, greater understanding of the bactericidal reaction depends upon studies at the cellular rather than the bulk phase level and this has been the trend in recent years. Salton (1951) has related the bactericidal activity of cetrimide against *Staphylococcus aureus* to its uptake by the cells, whilst Newton (1954) found that cell walls prepared from sensitive organisms absorbed several times as much polymyxin as those prepared from resistant organisms. Few & Schulman (1953) found marked differences in the absorption isotherms for polymyxin-sensitive and polymyxin-resistant organisms. The present communication represents a further attempt to relate the shape of isotherms describing the uptake of phenols by *Escherichia coli* to the bactericidal activity.

Experimental

MATERIALS

Bactericides. The phenolic bactericides were of Analar or Laboratory Reagent quality but were recrystallised or redistilled to the following characteristics. Phenol, m.p. 42·5°; *o*-chlorophenol, b.p. 175°; *m*-cresol b.p. 202°; chlorocresol, m.p. 65°; thymol, m.p. 49·5°; resorcinol, m.p. 109°; hexylresorcinol, b.p. 176°; benzylchlorophenol (5-chloro-2-hydroxydiphenylmethane), m.p. 47°.

Test organism. Escherichia coli (NCTC No 5933), maintained by freeze-drying from peptone solution. *Nutrient agar* for viable counts contained 1% peptone (Oxoid) and 0·5% NaCl gelled by 2% Kobé No 1 Agar, pH 7·4.

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METHODS

Evaluation of bactericidal activity. Freeze-dried *E. coli* were resuspended in distilled water, the suspension was passed through 3 successive 24-hr slope cultures, washed in sterile water and resuspended to a total count of 40×10^9 organisms per ml.

The reaction mixtures, containing $1{\cdot}4 \times 10^8$ *E. coli* per ml, were maintained at 25°. Samples were removed at intervals and diluted with $\frac{1}{4}$-strength Ringer solution; an appropriate number of standard drops of the dilution were added to 2·0 ml molten nutrient agar at 45°, rolled until solidified and incubated (48 hr) at 37°.

Estimation of uptake of phenols by E. coli. The method used was that described by Beckett, Patki & Robinson (1959a).

Preliminary experiments determined that a suspension of 45×10^9 *E. coli* per ml in 1% solution of phenol absorbed about 10% of the initial phenol. Experiments also revealed a negligible difference between the uptake by resting cells and the same number of freeze-dried cells resuspended in the phenol solutions; because of advantages in handling freeze-dried cells, they were used for the uptake studies.

A phenol solution of known concentration (2 ml) was added at 25° to 45×10^9 *E. coli* freeze-dried cells and equilibrated (10 min) at 25°. The cells were then centrifuged (10 min) at 8,500 *g* after which 1 ml supernatant was removed by means of a clean, dry Agla syringe. 0·5 ml was run into a 100 ml flask and made up to volume with phosphate buffer at pH 6·0. The solution was assayed spectrophotometrically at 270 mμ, using 4 cm cells. The reading (A) represented phenol + exudate.

A further 0·5 ml supernatant was shaken in a separator with 5 ml Analar chloroform and 5 ml phosphate buffer at pH 6·0, extraction being completed with 8×5 ml chloroform into which the phenol partitioned. The combined (40 ml) chloroform volumes were washed with 2×3 ml distilled water, the aqueous washings being added to the aqueous phase remaining in the separator.

The aqueous phase was then heated in a beaker to remove traces of chloroform, cooled, adjusted to 100 ml with buffer and assayed at 270 mμ. This reading (B) represented exudate.

A—B is then the actual phenol remaining (C) in the supernatant after contact with cells.

Chloroform used in the extractions was checked for zero absorbance at 270 mμ against water. Replicate assays of a given supernatant solution showed that estimates of residual phenol were reproducible within ±1·5% of the mean value obtained.

With the higher molecular weight compounds a smaller number of organisms per ml gave a measurable uptake, the actual density of cells used being recorded on the uptake isotherms.

Results

VIABLE COUNTS

An estimate of the overall accuracy of the viable counting technique was made by counting 20 roll tubes prepared from a suspension of *E.*

coli in $\frac{1}{4}$-strength Ringer solution. This gave a value of $\chi^2 = 12{\cdot}35$ which corresponds with P = 0·8–0·9. This was considered satisfactory.

The same test applied to *E. coli*/bactericide reaction mixtures indicated an increase in χ^2 values as the reaction proceeded. Mortalities in excess of 90% tended to produce high values of χ^2 which corresponded with low probabilities. High values of χ^2 may have been due to sampling reaction mixtures containing small numbers of randomly distributed organisms or to a tendency for survivors to aggregate in clumps. Errors in extinction time methods of evaluating bactericidal activity have in fact been attributed to sampling mixtures containing clumped organisms (Berry & Bean, 1954). For this reason, the activities of different bactericides were compared at a mortality level below 90%.

The probit mortality-log time regressions for the death of *E. coli* in each of the bactericides (not illustrated) had distinct "breaks" or inflexions between probits 4·5 and 5·5 as a result of which the regressions were bilinear; *t*-tests showed the slopes of the two parts of the regressions to be significantly different.

Analyses of variance showed the probit-mortality-log time regressions for 10 replicate experiments with one concentration of a phenol to be both linear and parallel for mortalities in excess of that at which the "break" occurred, but not parallel nor necessarily linear for mortalities below the "break." The phenols were therefore compared at a mortality level between 50 and 90%, the level ultimately selected being the mortality corresponding to probit 6·0, i.e. 84·13% (LT84).

COMPARATIVE BACTERICIDAL ACTIVITY OF DIFFERENT PHENOLS

The comparative bactericidal activities of the eight phenols are represented in Fig. 1 where log concentration is plotted against LT84. The regressions in Fig. 1 were extrapolated to intercept the abscissa at

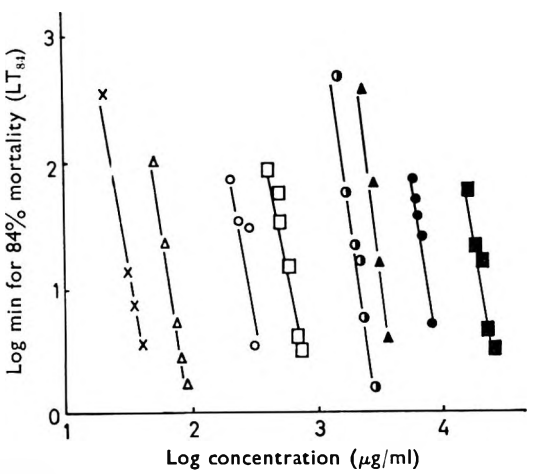

FIG. 1. Bactericidal activity of phenols against *E. coli*. ■, Resorcinol. ●, Phenol. ▲, *m*-Cresol. ◑, *o*-Chlorophenol. □, Chlorocresol. ○, Thymol. △, Hexylresorcinol. ×, Benzylchlorophenol.

a concentration equivalent log LT84 = 0. This represents a mortality of 84% in 1 min and we have called this the *maximum effective concentration* since more rapid reactions are difficult to assay with reasonable certainty. It was used as the basis for comparing the different bactericides.

UPTAKE OF PHENOLS BY *E. coli*

The uptake of phenols by the bacterial cells is represented by absorption isotherms which are shown in Fig. 2. Seven of the eight isotherms are similar in shape and appear to resemble most closely the type S isotherm of Giles, MacEwan, Nakhwa & Smith (1960). However, instead of being continuously curvilinear like the type S isotherms they are bilinear, with the proximal portion being almost perfectly linear. The initial concentration of the bulk solution (Table 1) which corresponded with the "break" or inflexion on the isotherm is calculated by summing the equilibrium concentration (abscissae in Fig. 2) at the inflexion and the amount that had been taken up by the cells (ordinates in Fig. 2). The shape of the absorption isotherm for resorcinol was quite different and resembled the Giles isotherm type L. No inflexion occurred in the resorcinol isotherm.

TABLE 1. RELATIONSHIP BETWEEN "MAXIMUM EFFECTIVE CONCENTRATION"* OF PHENOLS AND CONCENTRATION AT INFLEXION IN ABSORPTION ISOTHERM

Compound	Maximum effective concentration* µg/ml (A)	Concentration at inflexion in absorption isotherm µg/ml (B)	Ratio $\frac{A}{B}$
Resorcinol	$3{\cdot}44 \times 10^4$	—	
Phenol	$1{\cdot}18 \times 10^4$	$1{\cdot}20 \times 10^4$	0·98
m-Cresol	$4{\cdot}28 \times 10^3$	$3{\cdot}25 \times 10^3$	1·32
o-Chlorophenol	$3{\cdot}40 \times 10^3$	$3{\cdot}10 \times 10^3$	1·10
Chlorocresol	$1{\cdot}12 \times 10^3$	$1{\cdot}00 \times 10^3$	1·12
Thymol	$4{\cdot}33 \times 10^3$	$6{\cdot}00 \times 10^3$	0·72
Hexylresorcinol	$1{\cdot}25 \times 10^3$	$1{\cdot}06 \times 10^2$	1·18
Benzylchlorophenol	$5{\cdot}60 \times 10$	$5{\cdot}90 \times 10$	0·95
Mean			1·05

* Defined as "A mortality of 84% in 1 min."

Discussion

A linear probit-mortality-log time regression representing the death of bacteria in a bactericide indicates that the death-rate of the organisms is a function of their resistance. Departure from linearity may indicate a mixed bacterial population or that death is the result of more than one cause or mechanism. For example, in the early stages of the reaction between phenols and *E. coli*, cell exudates appear in the supernatant liquors (Bean & Walters, 1955) and at a later stage the opacity of the cells increases (Beckett, Patki & Robinson, 1959b).

Acceptance of the vitalistic explanation of the course of the bactericidal reaction ignores the observation that when the reaction between a phenol and *E. coli* causes a mortality of more than 99%, the survivors may multiply in what was initially a bactericidal system devoid of intentionally added nutrients (Bean & Walters, 1955). The nutrient material consists of

exudates which are produced during the normal metabolism of the cells: they appear in greater quantity however because of the action of the bactericide.

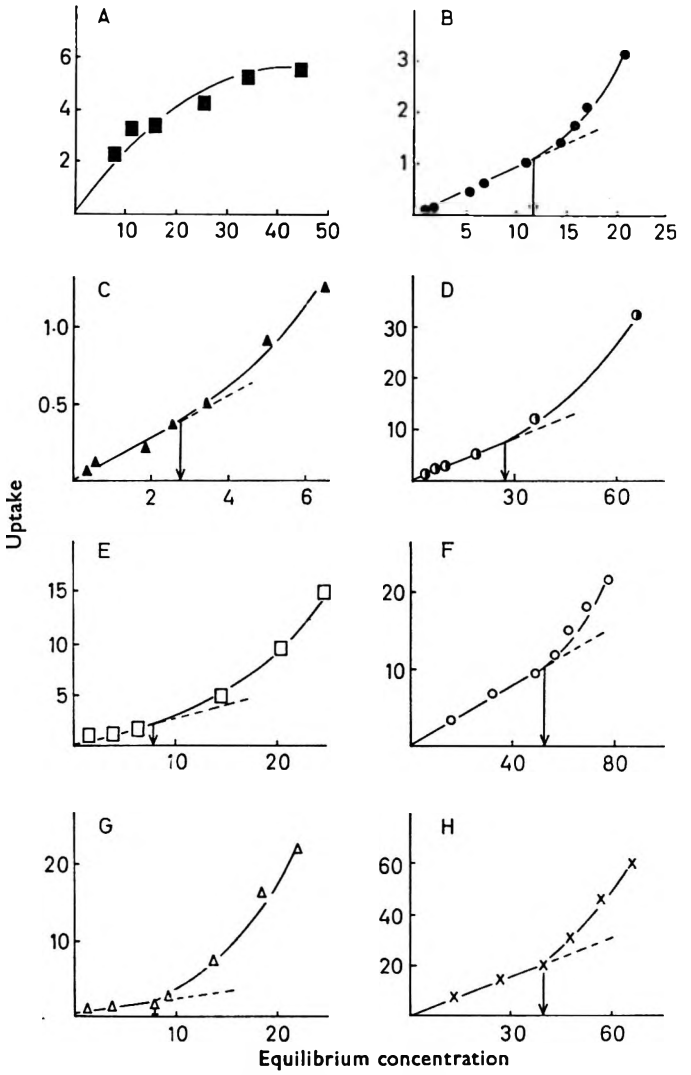

FIG. 2. Uptake of phenols by *E. coli*. A. Resorcinol (λ_{max} 274 mμ; ϵ 926·3). Equilibrium concentration and uptake both $\times 10^{-3}$ μg/ml, by 13×10^{11} *E. coli*. B. Phenol (λ_{max} 270 mμ; ϵ 1496·2). Equilibrium concentration and uptake $\times 10^{-3}$ μg/ml, by 45×10^{9} *E. coli*. C. *m*-Cresol (λ_{max} 271 mμ; ϵ 1445·3). Equilibrium concentration and uptake $\times 10^{-3}$ μg/ml, by 40×19^{9} *E. coli*. D. *o*-Chlorophenol (λ_{max} 273 mμ; ϵ 779·8). Equilibrium concentration and uptake $\times 10^{-2}$ μg/ml, by 65×10^{9} *E. coli*. E. Chlorocresol (λ_{max} 280 mμ; ϵ·1540·1). Equilibrium concentration and uptake $\times 10^{-2}$ μg/ml, by 20×10^{9} *E. coli*. F. Thymol (λ_{max} 274 mμ; ϵ 1830·6). Equilibrium concentration and uptake $\times 10^{-1}$ μg/ml, by 20×10^{9} *E. coli*. G. Hexylresorcinol (λ_{max} 279·5mμ; ϵ 2564·2). Equilibrium concentration and uptake $\times 10^{-1}$ μg/ml, by 4×10^{9} *E. coli*. H. Benzylchlorophenol (λ_{max} 282 mμ; ϵ 2230·5). Equilibrium concentration and uptake, μg/ml, by 2×10^{9} *E. coli*.

For each of the phenols employed in the present experiments, a plot of LT84 against logarithm of the concentration yielded a regression line which was approximately linear (Fig. 1), the slope of the line being the concentration exponent of the phenol. A good fit to linearity was held by earlier workers to indicate that the bactericidal reaction was a monomolecular reaction.

With the exception of that for resorcinol, the isotherms for the uptake of phenols by *E. coli* all resemble the type S isotherm of Giles & others (1960). The linear proximal sections indicate that the amount taken up is proportional to the initial concentration of the bulk aqueous phase and suggest an uptake mechanism which behaves as though the phenol partitioned between two phases, e.g. the external phase and biophase.

At a concentration which is a characteristic of each of the seven phenols, the absorption isotherms indicate a sudden marked increase in the uptake of phenol by the cells, and this continues to rise with increasing bulk concentration. With phenol itself this concentration is the same as the "protein precipitating concentration" recorded by Cooper (1912) and Cooper & Woodhouse (1923). Certainly, at this concentration an increase was noted in the opacity of the bacterial suspension, as indicating a precipitation or coagulation of bacterial proteins.

Giles & MacEwan (1957) describe an adsorption isotherm in which the initial portion is linear, and suggest it represents a condition in which the number of sites available for adsorption remains constant, even though the amount of solute adsorbed increases. They also suggest that this type of uptake occurs when the substrate structure is opened up by the solute, and liken the process of adsorption to the opening of a zip-fastener. Presumably when phenol enters a bacterial cell the hydrogen bonds of the cellular proteins are broken, permitting the unfolding of the chains and the uptake of a greater quantity of phenol. Evidence is offered by Putnam & Neurath (1944) of the unfolding of protein side-chains on the interaction of sodium dodecyl sulphate and serum albumin. In the bactericidal system this mechanism presumably continues up to the concentration represented by the point of inflexion in the absorption isotherm. Above this concentration phase separation takes place or a non-stoichiometric complex is formed (Putnam & Neurath, 1944).

With resorcinol the uptake isotherm resembles the type L isotherm of Giles & others (1960) and indicates a different mechanism or a different type of affinity for the bacterial cell. Undoubtedly the resorcinol isotherm is a reflection of its extreme water-solubility and therefore a different cell-water partition coefficient. Saturation of the bacterial cell is easily attained with resorcinol but not with the remaining seven phenols and this suggests a certain lack of affinity of the cellular lipoproteins for resorcinol.

COMPARISON OF UPTAKE DATA AND MAXIMUM EFFECTIVE CONCENTRATION

For each of the phenols—except resorcinol—the initial bulk concentration at which the inflexion was observed in the uptake isotherm is compared with the *maximum effective concentration* (Table 1). The approxi-

mate constant ratio of the *maximum effective concentration* and concentration of the point of inflexion on the uptake isotherm is in support of the observations of Cooper (1912; 1913) and Cooper & Woodhouse (1923) who record a relation between the bactericidal activities of several phenols and the concentrations at which they precipitate proteins. Cooper & Woodhouse also observed a change in the protein/water partition coefficient at the concentration at which they precipitate the protein, which again appears to be in line with the marked increase in the uptake of the phenols by the cells at the maximum effective concentration.

At approximately the concentration at which the inflexion appears on the phenol absorption isotherm, Loveday & James (1957) observed a change in the electrophoretic mobility of *Aerobacter aerogenes*. They interpreted this as indicating surface saturation of the cells by phenol. In view of the several independent observations on the relation between bactericidal activity and uptake by the cell of bactericide molecules, the point of inflexion on the uptake isotherms may be regarded as an iso-active point and a standard state for comparing bactericidal activity with drug uptake.

Loveday & James (1957) have shown that toxicity of phenols can be correlated directly with surface saturation of the cell and, from measurements of electrophoretic mobility, they have predicted the phenol coefficient of several phenols. Their conclusions that there is a correlation between uptake and bactericidal activity, together with the conclusions reached in this paper, suggest that the course of the bactericidal action may ultimately be explainable without recourse to either the classical monomolecular or vitalistic theories, both of which are open to serious objections.

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Effect of chlorhexidine upon ^{32}P release and cell viability in *Escherichia coli*

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The amount of the cold trichloroacetic acid soluble fraction (metabolic pool), expressed as a percentage of its ^{32}P content, released from labelled cells of *Escherichia coli* treated with chlorhexidine has been compared with the percentage of cells killed by the chlorhexidine. Treatment was with amounts varying from 0–64 μg/ml for 5 min at 20°. Approximately 100% of the cells were killed and 100% of the metabolic pool released by treatment with 64 μg/ml of chlorhexidine. At 4, 8 and 16 μg/ml the percentage of cells killed by chlorhexidine is significantly lower than the percentage of the metabolic pool released.

CHLORHEXIDINE causes the release of ^{32}P from labelled cells of *Micrococcus lysodeikticus* (Rye & Wiseman, 1964) and of pentose and material absorbing at 260 mμ from *M. lysodeikticus* (Wiseman, 1964), *Staphylococcus aureus* and *Escherichia coli* (Hugo & Longworth, 1964). The pattern of release is similar in all instances. As the concentration of chlorhexidine is increased, the extent of release increases to a maximum then decreases at higher concentrations.

Using chlorhexidine at concentrations up to that causing the maximum release of ^{32}P from *M. lysodeikticus*, Rye & Wiseman (1965) showed that the initial release came from the metabolic pool which is that fraction soluble in cold trichloroacetic acid. They suggested that if the ^{32}P content of this fraction was not completely released only a proportion of the cells were leaking.

Hugo & Longworth (1964) determined the mean single survivor times of *Staph. aureus* and *E. coli* after treatment with chlorhexidine concentrations very much greater than those producing maximum release. These authors concluded that over the range of concentrations examined there was no relationship between the amount of cellular constituents released and the number of organisms killed, although they recognised that such a relationship might be present at lower concentrations of chlorhexidine.

This paper reports an investigation of the relation between the extent of ^{32}P release from labelled cells of *E. coli* and the number of organisms killed after 5 min treatment at 20° with concentrations of chlorhexidine up to that causing maximum release.

Experimental

MATERIALS

Chlorhexidine diacetate. Imperial Chemical (Pharmaceuticals) Ltd.
Escherichia coli (NCTC 86).

Growth medium. Ammonium chloride 5×10^{-2}M, magnesium chloride 5×10^{-4}M, sodium sulphate 5×10^{-4}M, potassium dihydrogen phosphate 10^{-3}M, trishydroxymethylaminomethane 10^{-1}M, and glucose 1 mg/ml. The pH was adjusted to 7·7 using M hydrochloric acid.

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Glucose-free medium was growth medium from which glucose had been omitted. *Low-phosphate medium* was growth medium without added phosphate and contained approximately 4×10^{-5}M phosphate as impurity.

Egg yolk solution. The separated yolks from two eggs were mixed with 200 ml of Oxoid tryptone soya broth and 6 g of kaolin. The resulting suspension was clarified by centrifuging at 6,000 rpm for 10 min.

METHODS

Absorbance measurements were made with a 1 cm path length at 650 mμ using a Unicam SP500 spectrophotometer.

Preparation of ^{32}P-labelled bacterial cells. E. coli was grown at 37° with aeration by shaking at 120 throws/min. 100 ml of cells in the log phase (absorbance approx. 0·4) were harvested by filtration through an 8 cm diameter Oxoid membrane filter, washed with and then suspended in low-phosphate medium at 37°. 0·5 μc of ^{32}P orthophosphate (specific activity 5 c/mg phosphorus) was added followed 30 sec later by 5 ml of 10^{-1}M potassium dihydrogen phosphate. After a further 1 min the labelled suspension was filtered, washed with and then suspended in glucose-free medium at 20°. The absorbance was adjusted to 0·600 with more glucose-free medium.

Reaction mixtures. 5 ml samples of the labelled cell suspensions were added to tubes each containing 5 ml of suitable dilutions of chlorhexidine in glucose-free medium at 20°. Five min after adding the cells, samples were removed for radioactivity measurements and for viable counting. Two min intervals were allowed between inoculations to permit all measurements to be made after exactly 5 min reaction time.

Radioactivity measurements. 2 ml samples in duplicate of the reaction mixtures and of untreated cell suspensions were filtered through 30 mm Oxoid membrane filters and washed with 4 ml of phosphate buffer pH 7·0. The washing of samples was completed within 15 sec. The membranes were attached to flat aluminium planchets with "Durofix" adhesive, dried and the ^{32}P content determined by counting for 50 min or until 50,000 counts were recorded, using a Beckman "Lowbeta" automatic planchet counter. The radiochemical statistical error was less than 1% ($P = 0{\cdot}95$).

^{32}P content of the cold trichloroacetic acid soluble fraction (metabolic pool). Samples (2 ml) of bacterial suspensions were mixed with equal volumes of 10% trichloroacetic acid and maintained at 4° for 30 min. The radioactivity remaining in the cells after this treatment was measured using the method described above and the ^{32}P content of the metabolic pool determined by subtraction from the total cellular radioactivity.

Determination of the percentage of organisms killed. Samples (0·2 ml) of the reaction mixtures were transferred to 10 ml of egg yolk solution or to 10 ml of glucose-free medium. Suitable dilutions in tryptone-soya broth or glucose-free medium respectively were then counted by the pour plate method using Oxoid tryptone-soya agar and 24 hr incubation at 37°. The number of organisms killed was calculated and expressed as a percentage of the number of viable organisms in untreated suspensions.

Results and discussion

When *E. coli* is labelled by cultivation in a synthetic medium containing ^{32}P labelled phosphate, approximately 20% of the radioactivity is present in the metabolic pool (Roberts, Abelson, Cowie, Bolton & Britten, 1957). The method of labelling described in this paper produces cells in which 75–85% of the radioactivity is in the metabolic pool and thus enables changes in this pool to be more accurately measured. Fig. 1 shows the changes in the total cellular radioactivity and of that fraction insoluble in cold trichloroacetic acid over a period of 3 hr at 20° in untreated cells suspended in glucose-free medium. The decrease in the ^{32}P content of the metabolic pool is due either to leakage from the cells or to an exchange with non-labelled phosphate in the suspending medium. This rate of decrease (0·23%/min) is approximately linear over 3 hr.

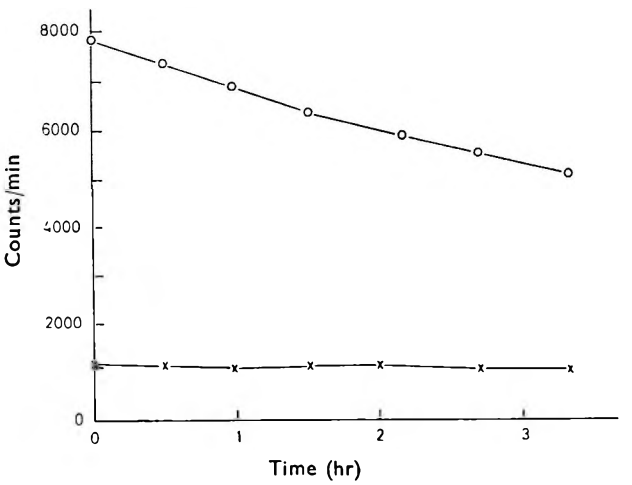

FIG. 1. The changes with time of the ^{32}P content of labelled cells of *Escherichia coli* suspended in glucose-free medium pH 7·7 at 20°. Cell concentration $3·2 \times 10^8$/ml. O——O, total cellular radioactivity. X——X, radioactivity remaining after treatment with 5% trichloroacetic acid for 30 min at 4°.

Fig. 2 shows the effect on the total cellular radioactivity when *E. coli* cells are treated with various concentrations of chlorhexidine. The amount of radioactive material released from the cells increases with chlorhexidine concentrations up to 64 μg/ml when it becomes constant and is approximately equal to the ^{32}P content of the metabolic pool.

Viable counts and ^{32}P release. Experiments to assess the efficiency of several agents for the inactivation of chlorhexidine showed that the highest recovery was obtained by using solutions of fresh egg yolk in tryptone-soya broth as the primary inactivator and tryptone-soya broth for the remaining dilutions.

Fig. 3 shows the average of six experimental determinations of the percentage of organisms killed after 5 min treatment with chlorhexidine, at concentrations up to 64 μg/ml, when both egg yolk and dilution were

used to inactivate the chlorhexidine. Fig. 3 also shows the extent of ^{32}P release expressed as a percentage of the metabolic pool after 5 min treatment with chlorhexidine. These results are the average of seven duplicate experiments. Table 1 shows the average of five experimental determinations of the apparent percentage of organisms killed after 5 min treatment with chlorhexidine when dilution only was used to inactivate the chlorhexidine.

TABLE 1. EFFECT OF TREATMENT WITH CHLORHEXIDINE FOR 5 MIN AT 20° UPON THE APPARENT VIABILITY OF *Escherichia coli*

Chlorhexidine concentration μg/ml	2	4	8	16	32	64
Apparent percentage of cells killed with estimated standard deviation	13 ± 6·5	32 ± 6·5	39 ± 12	80 ± 20	>99	>99

Suspending medium glucose-free medium pH 7·7. Cell concentration $3{\cdot}2 \times 10^8$/ml. Chlorhexidine inactivated by dilution alone.

At concentrations of chlorhexidine between 2 and 32 μg/ml the percentage of the metabolic pool released is significantly greater than the percentage of cells killed. A possible explanation is that when chlorhexidine is adsorbed by bacteria it produces cellular damage resulting initially in leakage of the metabolic pool and finally in death. The chlorhexidine-cell ratio probably governs both the rate at which this

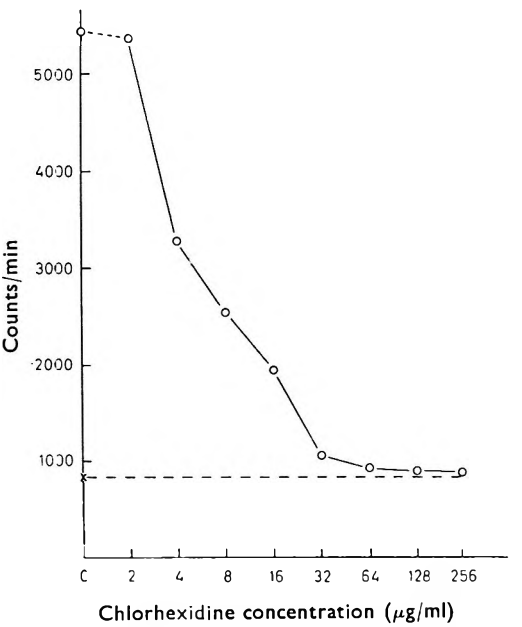

FIG. 2. The effect of treatment with chlorhexidine for 5 min at 20° on the ^{32}P content of labelled cells of *Escherichia coli* suspended in glucose-free medium pH 7·7. Cell concentration $3{\cdot}2 \times 10^8$/ml. ○——○, total cellular radioactivity. X——X, radioactivity remaining after treatment with 5% trichloroacetic acid for 30 min at 4°.

process occurs and the proportion of cells damaged. Thus at low chlorhexidine concentrations (4, 8 and 16 μg/ml), if the adsorbed bactericide molecules are removed or inactivated by egg yolk before death has occurred, then some of the cells which have lost all or part of their metabolic pool are able to recover. This interpretation recalls that of Judis (1962) who studied the action of phenolic disinfectants on *E. coli* and suggested that the leakage of cellular constituents probably precedes the death of the organisms and that a certain amount of damage to the cell membrane can be tolerated and repaired.

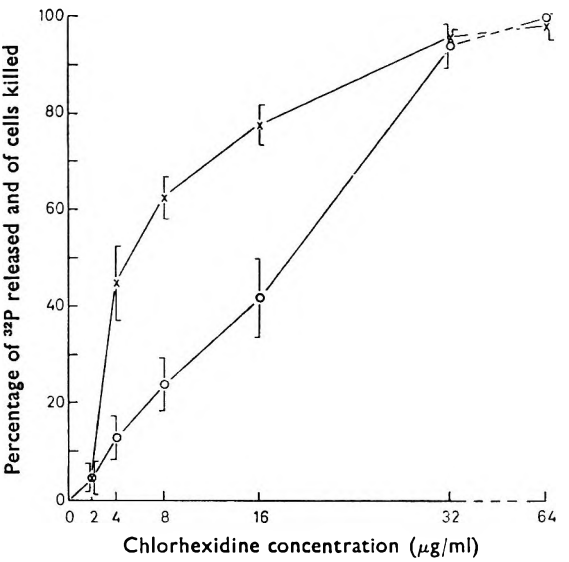

FIG. 3. Effect of chlorhexidine after 5 min at 20° on the release of ^{32}P from, and the cell viability of *Escherichia coli* suspended in glucose-free medium pH 7·7. Cell concentration $3{\cdot}2 \times 10^8$/ml. X——X, ^{32}P release as a percentage of the metabolic pool. O——O, percentage of cells killed. The results are the mean of six experiments and estimated standard deviations are indicated.

Within 5 min at higher concentrations of chlorhexidine (32 and 64 μg/ml) most of the cells appear to be damaged beyond recovery; little increase in the percentage of survivors recovered is observed on inactivating the chlorhexidine with egg yolk.

Acknowledgement. The authors are grateful to Mrs. S. Kaye for valuable technical assistance.

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Screening for tertiary and quaternary alkaloids in some African *Fagara* species

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A method has been described for the separate extraction of tertiary and quaternary alkaloids from plant material. Different thin-layer chromatographic systems have been used to separate compounds within each group of alkaloids. The methods have been applied to the screening of the barks of five *Fagara* species in which the presence of angoline, angolinine, skimmianine and, in some cases, 1-hydroxy-2,9,10-trimethoxy-*NN*-dimethylaporphinium chloride is indicated.

IN recent research on alkaloids of the family Rutaceae interest has attached to the genus *Fagara* because of an attempt to differentiate it chemotaxonomically from the closely related *Zanthoxylum* (Price, 1963). Failure to clarify the taxonomic position was largely the result of inadequate information on the types of alkaloids present in both genera. The botanical classification of many of the plants investigated is confused, particularly of the Asiatic species some of which Engler (Engler & Prantl, 1931) transferred from the *Zanthoxylum* to the *Fagara*; this transfer has not been recognised in much of the subsequent chemical literature. The genus *Fagara* contains approximately 250 species several of which have been reported to contain alkaloids (Table 1).

In the present work barks of the African species *F. leprieurii* Engl., *F. macrophylla* Engl., *F. viridis* A. Cheval. and *F. xanthoxyloides* Lam., all previously known to contain tertiary alkaloids (Table 2), and of *F. chalybea* Engl., not previously investigated, were examined for both tertiary and quaternary alkaloids.

Experimental

MATERIALS

The stem and root barks of *F. chalybea* Engl. were collected in Kenya, and those of *F. leprieurii* Engl., *F. macrophylla* Engl., *F. viridis* A. Cheval., and *F. xanthoxyloides* Lam. were collected in Nigeria: all were authenticated at source and supplied by The Tropical Products Institute, London.

Alumina for thin-layer chromatography (Camag). The plates (0·25 mm) were activated (3 hr) at 120° and stored in a desiccator for not more than five days.

Cellulose for thin-layer chromatography (Whatman Chromedia CC 41). The plates (0·25 mm) were stored in the absence of a desiccant.

Chloroform (ethanol-free and dried).

Developing solvents: 1(a) ethanol–chloroform (2:98); 1(b) ethanol–chloroform (4:96); 2(a) n-butanol–glacial acetic acid–water (10:1:3) (Giacopello, 1965); 2(b) t-amyl alcohol–isoamyl alcohol–formic acid–water (1:1:1:5) (Rafauf, personal communication).

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ISOLATION OF ALKALOIDS

The powdered stem (or root) bark (25 g) was extracted in a Soxhlet apparatus with light petroleum (bp 40–60°), then chloroform and, finally, ethanol (95%). The residue left on evaporation of the ethanol was extracted with hydrochloric acid (5%, 3×10 ml) and the acid solution extracted with chloroform (Gilbert & others, 1965) in a liquid-liquid extractor until extraction of alkaloids ceased. The aqueous phase was

TABLE 1. DISTRIBUTION OF TERTIARY AND QUATERNARY ALKALOIDS IN THE GENUS *Fagara*

Species	Alkaloids		Reference
	Tertiary	Quaternary	
South American			
F. coco Engl.	+	+	Comin & Deulofeu, 1954
F. hyemalis Engl.	−	+	Albonico, Kuck & Deulofeu, 1964
F. naranjillo Engl.	−	+	*Ibid.*
F. nigrescens Fries	−	+	*Ibid.*, Kuck, 1966
F. pterota Engl.	−	+	Albonico, Kuck & Deulofeu, 1964
F. rhoifolia Engl.	−	+	*Ibid.*
" "	+	+	Calderwood & Fish, 1966
F. tingoassuiba Hoehne	+	+	Riggs, Antonaccio & Marion, 1961
Australian			
F. brachyacantha Engl. (*Z. brachyacanthum* F. Muell.)	+	+	Jowett & Pyman, 1913
F. veneneficа Engl. (*Z. veneneficum* F. M. Bailey)	+	+	Cannon, Hughes, Ritchie & Taylor, 1953
Asiatic			
F. ailanthoides Engl. (*Z. ailanthoides* Sieb. & Zucc.)	+	+	Tomita & Ishii, 1958
F. avicennae Lam. (*Z. avicennae* (DC.)	−	+	Arthur, Hui & Ng, 1959
F. hamiltoniana Engl. (*Z. hamiltonianum* Wall.)	−	+	Gopinath, Kohli, Khan & Kidwai, 1963
F. nitida Roxb. (*Z. nitidum* DC.)	+	+	Arthur, Hui & Ng, 1958
F. oxyphylla R. & C. (*Z. oxyphyllum*)	+	−	Chatterjee & Mukherjee, 1964
F. rhetsa Roxb. (*Z. rhetsa* DC.)	+	+	Chatterjee, Bose & Ghosh, 1959
F. schinifolia Engl. (*Z. schinifolium* Sieb. & Zucc.)	+	−	Ishii, 1961
F. semiarticulate St. John & Hosaka	+	+	Scheuer, Charg & Swanholm, 1962
African			
F. angolensis Engl.	+	−	Palmer & Paris, 1955
F. heitzii A. & P.	+	−	Palmer, 1956
F. leprieurii Engl.	+	−	*Ibid.*
F. macrophylla Engl.	+	−	*Ibid.*
F. melanacantha Engl.	+	+	*Ibid.*
F. parvifolia A. Cheval.	+	−	*Ibid.*
F. pubescens A. Cheval.	+	−	*Ibid.*
F. viridis A. Cheval.	+	−	Paris & Moyse-Mignon, 1948
F. xanthoxyloides Lam.	+	−	Paris & Moyse-Mignon, 1947

NOTE: Since this paper was submitted additional information on quaternary alkaloids of several South American *Fagara* species•, including *F. chiloperone* var. *angustifolia* Engl., has been published by Kuck, A. M. & Albonico, S. M. (1966). *Chemy Ind.*, 945–946.

basified (pH 8·0) with ammonia and re-extracted with chloroform until any residual tertiary alkaloids were removed. Finally the alkaline aqueous phase, still strongly alkaloid-positive, was extracted with n-butanol in a liquid-liquid extractor until free from alkaloids (Raffauf, personal communication).

TABLE 2. TERTIARY ALKALOIDS OF FOUR AFRICAN *Fagara* SPECIES

Species	Alkaloid	Reference
F. leprieurii Engl.	†Angoline †Angolinine Skimmianine	Palmer (1956)
F. macrophylla Engl.	Fagaramide †Fagaridine Skimmianine •†Un-named alkaloid •†Xanthofagarine	Goodson (1921) Paris & Moyse-Mignon (1951) Palmer (1956) King, Housley & King (1954) Paris & Moyse-Mignon (1951)
F. viridis A. Cheval.	Skimmianine	Paris & Moyse-Mignon (1948)
F. xanthoxyloides Lam.	†Artarine Fagaramide n-Isobutyldecenamide Skimmianine	Giacosa & Monari (1887) Thoms & Thümen (1911) Bowden & Ross (1963) Paris & Moyse-Mignon (1947)

† Alkaloids of unknown structure.
• Probably identical.

CHROMATOGRAPHY

The dried residues from each solvent extract were separately dissolved in chloroform (3 ml) or, in the case of the butanol extract, in ethanol (3 ml) and the solutions chromatographed, two-dimensionally, on alumina using the solvent systems 1(a) and 1(b) at 25°. The development distance was 10 cm for each solvent.

The butanol extracts were also chromatographed, two-dimensionally, on micro-granular cellulose using the developing solvents 2(a) and 2(b). Before each development the plates were equilibrated for 1 hr in the presence of the appropriate solvent. Development for a distance of 15 cm in each direction required approximately 120 and 140 min, respectively, at a constant temperature of 25°.

The following alkaloids were used as markers on both types of chromatoplates: α-allocryptopine, angoline, angolinine, 1-hydroxy-2,9,10-trimethoxy-*NN*-dimethylaporphinium chloride, α-(−)-*N*-methylcanadine chloride, (+)-tembetarine chloride and skimmianine.

Plates were examined in daylight and at 366 mμ; they were then sprayed with modified Dragendorff's reagent. The cellulose plates were sprayed first with ferric chloride reagent (1%, in ethanol 95%) to detect phenolic alkaloids (greyish-green to green areas) then with Dragendorff's reagent.

TABLE 3. IDENTIFICATION OF ALKALOIDS ON TWO-DIMENSIONAL ALUMINA CHROMATOGRAMS

Compound	Average Rf values		Colour	
	Solvent 1(a)	Solvent (1)b	In daylight	In ultraviolet light
All quaternary alkaloids	0·00	0·00		
Tertiary alkaloids				
A	0·06	0·13	red	dark purple
Angolinine	0·05	0·46		greenish-yellow
B	0·16	0·55		blue
C	0·53	0·55		
D	0·76	0·79		
Skimmianine	0·83	0·87		violet
Angoline	0·95	0·82	yellow	intense yellow
E•	0·88	0·97		pale blue

• Probably fagaramide.

Relative amounts of alkaloids were estimated by the size and colour density of the alkaloid areas after final spraying; results are given in Tables 3, 4 and 5.

TABLE 4. RELATIVE AMOUNTS OF TERTIARY ALKALOIDS IN BARKS OF *Fagara* SPECIES

	F. chalybea		*F. leprieurii*		*F. macrophylla*		*F. viridis*		*F. xanthoxyloides*	
Compound	stem	root	stem	root	stem	root	stem	root	stem	root
A			+	++	+	+	++	++		+
Angolinine ..	+	++++ ++	++++	++++ ++	+	++	+	+		+++
B		+	+		+	+++		+		+
C		+								++
D		+								
Skimmianine ..	+++	++++ ++	+	++++ ++	+	++++	++++ ++	++++ +	+++	++
Angoline ..	++++	++++ ++++	++++	++++ ++++	++	++++	++++	++++	++	+++
E•		+(?)				++				++

• Probably fagaramide.

Results and discussion

The extraction procedure removed both tertiary and quaternary alkaloids from all five *Fagara* species. Most of the tertiary bases were extracted by light petroleum and chloroform; the former extracted all base E (probably fagaramide) and partially extracted angoline, angolinine and skimmianine, while chloroform extracted most of the remainder of these latter alkaloids, together with bases designated A, B, C and D. The ethanol extract contained mainly quaternary compounds with the small amount of remaining tertiary bases. These tertiary bases, together with traces of quaternary alkaloids, were removed from the dried ethanol extract by extraction with chloroform, firstly from an acidic and later from an alkaline aqueous phase. The first extraction removed most of the alkaloids and gave a much cleaner chloroform solution than was subsequently obtained in the alkaline extraction. A final extraction of the alkaline phase with n-butanol yielded a solution containing only the quaternary alkaloids.

Chromatography on alumina distinguished between tertiary and quaternary alkaloids; under the conditions used, the latter remained at the point of application while all tertiary bases moved and excellent separation of these was obtained on two-dimensional chromatograms. With cellulose powders containing binding agents no separation of quaternary alkaloids was obtained; only continuous streaks of alkaloid were observed. Micro-granular cellulose plates, prepared without a binding agent (Giacopello, 1965), gave excellent separation of these compounds.

Fagara leprieurii, *F. macrophylla*, *F. viridis* and *F. xanthoxyloides* all previously known to contain tertiary bases, were shown to contain significant amounts of quaternary alkaloids; *F. chalybea*, not previously

TABLE 5. RELATIVE AMOUNTS OF QUATERNARY ALKALOIDS IN BARKS OF *Fagara* SPECIES

	Average Rf values†	*F. chalybea*		*F. leprieurii*		*F. macrophylla*		*F. viridis*		*F. xanthoxyloides*	
Compound	Solvent 2(a) 2(b)	stem	root	stem	root	stem	root	stem	root	stem	root
Q1	0·27, 0·12			+	+	+	+	+	+		
Q2*	0·39, 0·21			+++	+++	+	+	+++	+++	++	+
Q3*	0·38, 0·28	+	+	+++	+++		++	+++	+++	++++	++
Q4*	0·46, 0·30		+	++		+				+++	++
Q5*	0·46, 0·36	+++	++	+++	++	++		+	+++		
Q6	0·36, 0·35										+++
Q7	0·48, 0·37	++++	++++	++++	+++	++++	++++	+++	+++	+++	++++
Q8	0·45, 0·47							++++	++++		
Q9	0·52, 0·50					+	++				
Q10	0·56, 0·53	+	++	++	++	++++	++++	++	+++	++	++
Q11	0·59, 0·68									+	++
Q12	0·71, 0·74	+	+	+++	++	+	++	+	+	++	+

Q2 Probably 1-hydroxy-2,9,10-trimethoxy-*NN*-dimethylaporphinium chloride.
Q4 Probably (+)-tembetarine chloride.
* Phenolic alkaloids.
† Obtained from two-dimensional cellulose chromatograms using solvents 2(a) and 2(b).

investigated, was also shown to contain both types of compound. In most instances the root bark contained a larger total amount of alkaloid and a larger number of individual alkaloids than the stem bark of the same species (Tables 4 and 5).

TERTIARY ALKALOIDS

On thin-layer chromatoplates, all five species showed the presence of three principal tertiary bases corresponding to angoline, angolinine and skimmianine, though the amount of angolinine in *F. viridis* was small. The furoquinoline base skimmianine is the most common alkaloid not only in the genus *Fagara* but also in the family Rutaceae. Appreciable amounts of alkaloid A were found in all species except *F. chalybea*. The remaining tertiary bases, B, C, D and E were of minor importance and their distribution varied amongst the five species. The tertiary protopine base α-allocryptopine, reported in the two Australian species *F. brachyacantha* and *F. venenefica* and in the two South American species *F. coco* and *F. rhoifolia* (Table 1), appeared to be absent from these African species.

QUATERNARY ALKALOIDS

Quaternary alkaloids (unidentified) have previously been reported in but one African *Fagara* species, *F. melanacantha* (Palmer, 1956). We have now found such compounds as a major group of alkaloids in both the stem and root barks of the species investigated. Some of the quaternary alkaloids are phenolic and one of these appeared to be identical with 1-hydroxy-2,9,10-trimethoxy-*NN*-dimethylaporphinium chloride. When an authentic sample was admixed with the butanol extracts, from all species except *F. chalybea* there resulted an area of increased intensity for the spot Q2 (Table 5). This compound has been reported previously in the two South American species *F. tingoassuiba* (Riggs, Antonaccio & Marion, 1961) and *F. rhoifolia* (Calderwood & Fish, 1966). The compound Q4, found in *F. chalybea*, *F. leprieurii* and *F. xanthoxyloides*, appears to be identical with the quaternary benzylisoquinoline alkaloid (+)-tembetarine chloride, previously isolated from the South American species *F. naranjillo*, *F. hyemalis*, *F. nigrescens*, *F. pterota* and *F. rhoifolia* (Albonico, Kuck & Deulofeu, 1964). The quaternary protoberberine base α-(−)-*N*-methylcanadine chloride found in *F. rhoifolia* (Calderwood & Fish, 1966), *F. brachyacantha* (Jowett & Pyman, 1913) and in *F. venenefica* (Cannon, Hughes, Ritchie & Taylor, 1953) did not correspond, on two-dimensional chromatograms (Rf values 0·68, 0·71), with any of the quaternary alkaloids of the barks examined.

The five African *Fagara* species examined contain certain tertiary and quaternary alkaloids in common, but of the various related chemical groups of alkaloids known to be present in the genus *Fagara*, not all appear to be represented in these African species.

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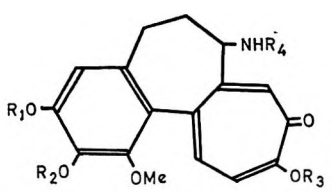
Phytochemical investigations of some species of *Colchicum*

K. R. FELL AND DOREEN RAMSDEN

A colorimetric assay for colchicine and its congeners has been devised. It is based on the yellow colour produced by the treatment of these alkaloids with mineral acid and is useful over the range 0·0005–0·0025% w/v. Together with the method of Pesez (1957) it has been used to assay extracts of species of *Colchicum* and the individual alkaloids were identified by paper chromatography. It was concluded that *C. :unicatum*, a native of Israel, contained sufficient colchicine to make it a useful source of this alkaloid.

THE presence of colchicine and many congeners in numerous species of *Colchicum* and other liliaceous plants was reported by Šantavý (1957). The alkaloids which contain the tropolonic ring C are either of the colchicine-type (I; R_3 = Me) or the colchiceine-type (I; R_3 = H). Each class in turn may be basic, neutral or phenolic.

Species of *Colchicum* indigenous to Israel have been reported to contain large amounts of colchicine (Boyko, 1954) and several have been investigated previously. Šantavý, Černoch, Lang, Malinský & Zajíčková (1951) isolated and assayed colchicine (I; neutral) and demecolcine (I; basic) from the corms of *C. cilicum* Hayek [= *C. steveni* Kunth (Stefanoff, 1926)] whilst Kaul, Moza, Šantavý & Vrublovský (1964), using paper chromatography, reported the presence of tropolonic alkaloids in *C.*



I

Alkaloid	R_1	R_2	R_3	R_4
Colchicine	Me	Me	Me	COMe
Colchiceine	Me	Me	H	COMe
Demecolcine	Me	Me	Me	Me
Substance C	H	Me	Me	COMe
Substance E_1	Me	H	Me	COMe
Substance B	Me	Me	Me	CHC
Substance S	Me	H	Me	Me

ritchii R. Br. *C. hierosolymitanum* Feinbr. has also been investigated and the amount of colchicine determined (Weizmann, 1952; Šantavý, Hoščálková, Podivínský & Potěšilová, 1954; Šantavý, Zajíček & Nemečková, 1957).

The aim of our investigation was threefold: (1) to evolve a rapid, colorimetric assay for tropolonic alkaloids of both the colchicine- and

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colchiceine-types; (2) to determine the amount of alkaloids in some species of *Colchicum* from Israel; (3) to identify the individual alkaloids of both classes by paper chromatography. As Israeli samples were small, samples of more readily-available species of *Colchicum* were included to ascertain that the assay procedures used were of value with only one small sample of the drug.

MATERIALS

Specimens of *C. ritchii* R. Br., *C. steveni* Kunth and *C. tunicatum* Feinbr. were received from Prof. M. Evenari and Dr. N. Feinbrun, the Hebrew University, Jerusalem, Dr. J. Galil, Tel-Aviv University, and the Tropical Products Institute, London. These were raised in a heated greenhouse. *C. ritchii* and *C. tunicatum* did not flower and leafy plants were used. *C. steveni* produced healthy, flowering plants and these were used whole. Corms of *C. autumnale minor* and *C.* hybrid Disraeli, from a bulb-grower, were raised in a garden in Bradford; plants in flower were used. *C. autumnale* L. was a commercial sample of colchicum corm. It contained much corm scale and other extraneous material. When assayed by the method of the *British Pharmacopoeia* (1963) it yielded only 0·20% of the alkaloids of colchicum corm (official limits: not less than 0·25%). *C. luteum* Bak. was donated by Prof. G. E. Trease, the University, Nottingham, and consisted of corms, devoid of scales, dried at a temperature sufficient to gelatinise the starch.

Phytochemical investigations

EXTRACTION PROCEDURES

Plants were harvested, washed and dried (30°). The material was then divided into flowering tops and underground organs or left whole, and then reduced to a coarse powder.

The powder (5 g) was extracted (3–4 hr) in a Soxhlet with ethanol (90%) (150 ml). Ethanol was evaporated under reduced pressure (60°) and the residue dissolved in distilled water and the solution filtered.

The solution was shaken with ether (6 × 20 ml), made just acid with dilute hydrochloric acid and shaken with chloroform (2 × 20 ml), made alkaline with concentrated ammonia solution and extracted with chloroform (6 × 20 ml). At this stage, all tropolonic alkaloids had been removed from the aqueous phase which did not give a lemon-yellow colour with concentrated hydrochloric acid.

Ether fractions were bulked, washed with distilled water (20 ml) and hydrochloric acid (5% v/v) (20 ml); the ether solution was then discarded.

Chloroform fractions were bulked, washed with distilled water (20 ml), hydrochloric acid (5% v/v) (20 ml), distilled water (20 ml) and aqueous sodium carbonate (10% w/v) (20 ml). The residual chloroform solution containing neutral and phenolic alkaloids was evaporated to dryness (100°), the residue dissolved in ethanol (2 ml) and again evaporated to dryness.

The acid washings from the ether and chloroform solutions containing basic alkaloids were combined, neutralised with solid sodium bicarbonate

and extracted with chloroform (2 × 20 ml), which removed all tropolonic alkaloids from the aqueous layer. The chloroform solution was evaporated to dryness (100°), the residue dissolved in ethanol (2 ml) and again evaporated to dryness.

The residues containing (i) basic and (ii) neutral and phenolic alkaloids were dissolved in ethanol (2 ml) for paper chromatography and in distilled water and made to 50 ml for colorimetric assay.

PAPER CHROMATOGRAPHY OF EXTRACTS

Whatman No. 1 paper and ascending technique were used. Papers were examined in ultraviolet light (360 mμ) and then treated with the vapour from concentrated hydrochloric acid or fuming nitric acid which coloured the spots of tropolonic alkaloids lemon-yellow. There has been no reference to the use of this spray reagent but Giebelmann (1964) used a spray of Millon's Reagent for colchicine and the yellow colour of the spots was presumably due to the concentrated nitric acid present.

Authenticated specimens of colchicine,* demecolcine† and colchiceine were used as reference substances. Solvent systems used, spot colours and Rf values are listed below.

A. Distilled water (method of Salo, 1960): Rf values and spot colours found for colchicine, demecolcine, colchiceine were respectively 0·71–0·82 (yellow in ultraviolet light and after spray), 0·37–0·49 (green-blue in ultraviolet light, yellow after spray), 0·59–0·64 (yellow in ultraviolet light and after spray). Salo (1960) quoted Rf values for colchicine of 0·7–0·8 and for demecolcine of 0·4–0·5.

B. n-Butanol-acetic acid-water (4 : 1 : 5) (method of Delong, Havrliková & Šantavý, 1955): Rf values and spot colours found; colchicine, colchiceine (yellow in ultraviolet light and after spray) travelled with solvent front (diffuse band); demecolcine (greenish-yellow in ultraviolet light, yellow after spray) was 0·70–0·78. Delong & others (1955) quoted 0·79 for demecolcine.

COLORIMETRIC ASSAYS OF EXTRACTS

Method of Pesez (1957). Into each of 6 tubes was placed 5, 4, 3, 2, 1, 0 ml of aqueous alkaloid (0·001% w/v) and each was diluted to 5 ml with water. To each tube was added aqueous isoniazid (10% w/v) (2 ml) and aqueous sodium carbonate (10% w/v) (1 ml). The tubes were heated (10 min) to 100°. The absorbance of the resulting deep orange-yellow solution was measured at 450 mμ, using the tube without alkaloid to set zero absorbance, in a Bausch & Lomb Spectronic 20 colorimeter. A calibration graph was drawn for colchicine and demecolcine. Extracts were treated similarly, after suitable dilution with water, and readings compared with the standard curve. The method assays alkaloids of the colchicine-type.

Proposed method. The lemon-yellow colour produced by treating colchicine with mineral acid (Hübler, 1865) arises from the presence of

* B.D.H. Laboratory Reagent.
† Colcemid CIBA Laboratories.

the tropolone ring C (Pauson, 1955). With colchicine and demecolcine we found that the absorbing species has a λmax 385 mμ and when the colour is produced under standard conditions the absorbance at this wavelength is directly proportional to the amount of alkaloid present. The linear relationship holds over the range 0·0005–0·0025% w/v alkaloid.

Into each of 6 tubes was placed 5, 4, 3, 2, 1, 0 ml aqueous alkaloid (0·005% w/v) and each was diluted to 5 ml with water. To each tube was added hydrochloric acid (s.g. 1·18) (5 ml) and the absorbance of the lemon-yellow colour, which developed immediately, was measured, using the tube without alkaloid to set the zero absorbance. A calibration curve was drawn for colchicine and demecolcine. Extracts were treated similarly, after suitable dilution with water, and the readings compared with those of the standard curve. This estimates those alkaloids of the Liliaceae which contain a tropolone ring (Šantavý, 1957) whether of the colchicine, or colchiceine type. Thus the difference between the assay figures given by the two methods may be taken as the amount of colchicine-type alkaloids present.

Results

Assay figures and alkaloids identified by paper chromatography are presented in Table 1.

By the paper chromatographic method of Salo (1960) a spot of Rf 0·009–0·34, fluorescing blue in ultraviolet light was observed. This is a very wide range of values but, as comparison with the chromatograms obtained by the method of Delong & others (1955) showed only one blue, fluorescent spot of Rf 0·82–0·84, it was considered that only one substance was present and that this was Substance C (I; R_1 = H, R_2 = R_3 = Me, R_4 = COMe) (phenolic) reported by Delong & others (1955) to have an Rf 0·84 and to fluoresce bright blue on paper chromatograms. Water is a poor solvent system for this substance.

Discussion

We have calculated from the results of Šantavý & others (1954) that, in the corms of *C. autumnale*, of all neutral and phenolic alkaloids containing the tropolone ring, 90% is colchicine, whilst 98% of the basic alkaloids is demecolcine. Other tropolonic alkaloids have been detected in species of *Colchicum*, but for most species substances isolated or detected have been colchicine, colchiceine, demecolcine and Substance C and/or E_1 (I; R_1 = R_3 = Me, R_2 = H, R_4 = COMe) (phenolic) and more rarely Substance B (I; R_1 = R_2 = R_3 = Me, R_4 = CHO) (neutral) and Substance S (I; R_1 = R_3 = R_4 = Me, R_2 = H) (basic/phenolic but found in basic fractions). The average amount of C and/or E_1 is 0·0007–0·0033% and so an assay for tropolonic alkaloids is virtually one for colchicine and demecolcine. This was verified by paper chromatography where the only tropolonic compounds detected were colchicine, colchiceine, demecolcine and Substance C. The colour with the spray was only prominent for colchicine and demecolcine, showing that these

TABLE 1. RESULTS OF COLORIMETRIC ASSAYS AND PAPER CHROMATOGRAPHY OF EXTRACTS OF SPECIES OF *Colchicum*.*

Plant and state	Colorimetric assays: Neutral/phenolic alkaloids: Tropolonic (Present method)		Colorimetric assays: Neutral/phenolic alkaloids: Colchicine type (Pesez (1957))	Colorimetric assays: Basic alkaloids: Troplonic (Present method)	Colorimetric assays: Basic alkaloids: Colchicine type (Pesez (1957))	Alkaloids identified by paper chromatography
C. autumnale L. (Colchicum corm)						Colchicine AB**
Mean	0·101	(12)†	0·097	0·098	0·095	Colchiceine B
Standard deviation	0·006		0·009	0·006	0·005	Substance C AB
Mean colchiceine-type alkaloids		0·004		0·003		Demecolcine AB
						Colouring matter A
C. autumnale minor corms						
Mean	0·080	(8)	0·079	0·106	0·101	Colchicine AB
Standard deviation	0·009		0·005	0·005	0·009	Colchiceine B
Mean colchiceine-type alkaloids		0·001		0·005		Substance C AB
						Demecolcine AB
Flowering tops						
Mean	0·304	(8)	0·282	0·122	0·103	Colchicine AB
Standard deviation	0·043		0·012	0·003	0·014	Colchiceine AB
Mean colchiceine-type alkaloids		0·022		0·038		Substance C AB
						Demecolcine AB
						Colouring matter A
Whole plant in flower						
Mean	0·112	(8)	0·091	0·092	0·087	Colchicine AB
Standard deviation	0·003		0·001	0·003	0·003	Colchiceine AB
Mean colchiceine-type alkaloids		0·021		0·005		Substance C AB
						Demecolcine AB
						Colouring matter AB
C. hybrid Disraeli corms						
Mean	0·032	(6)	0·028	0·034	0·032	Colchicine AB
Standard deviation	0·002		0·001	0·003	0·002	Colchiceine AB
Mean colchiceine-type alkaloids		0·004		0·002		Substance C AB
						Demecolcine AB
Flowering tops						
Mean	0·347	(2)	0·323	0·095	0·093	Colchicine AB
Standard deviation	0·012		0·004	0·006	0·006	Colchiceine AB
Mean colchiceine-type alkaloids		0·024		0·002		Substance C AB
						Demecolcine AB
						Colouring matter A
Whole plant in flower						
Mean	0·057	(6)	0·040	0·039	0·033	Colchicine AB
Standard deviation	0·002		0·002	0·001	0·002	Colchiceine AB
Mean colchiceine-type alkaloids		0·017		0·006		Substance C AB
						Demecolcine AB
						Colouring matter A
C. luteum Bak.						
Mean	0·049	(6)	0·047	cannot be	0·004	Colchicine AB
Standard deviation	0·002		0·003	assayed	0·001	Colchiceine AB
Mean colchiceine-type alkaloids		0·002		by this method		Substance C B
						Colouring matter A
						13 unidentified substances
C. ritchii R. Br. whole, leafy plant	0·055	(1)	0·050	0·060	0·056	Colchicine AB
Colchiceine-type alkaloids		0·005		0·004		Colchiceine AB
						Substance C AB
						Demecolcine AB
C. steveni Kunth whole, flowering plant	0·074	(1)	0·068	0·033	0·030	Colchicine AB
Colchiceine-type alkaloids		0·006		0·003		Colchiceine AB
						Substance C AB
						Demecolcine AB
C. tunicatum Feinbr. whole, leafy plant	0·160	(1)	0·155	0·56	0·050	Colchicine AB
Colchiceine-type alkaloids		0·005		0·006		Colchiceine AB
						Substance C AB
						Demecolcine B
						Colour matter A

(See next page for footnotes)

are the only tropolonic compounds present in large quantities, except in flowering tops where large amounts of colchiceine were detected.

There was only sufficient for one determination of each Israeli sample, therefore this work can constitute only a preliminary investigation. However, both assay methods used gave reasonably consistent results with other species of *Colchicum*. Those for *C. autumnale*, *C. autumnale minor* corms and *C. luteum* show good correlation with published results (Šantavý & others, 1951, 1954; Delong & others, 1955; Šantavý, 1957; Yusupov & Sadykov, 1962). There was an exception with the flowering tops of *C. autumnale minor* which show a large standard deviation when assayed by our method. The results were much lower than those quoted for the flowers of *C. autumnale* (Šantavý & Mačák, 1954) and in the basic fraction we found that the major alkaloid was demecolcine whereas Šantavý & Mačák (1954) quoted Substance S.

C. hybrid Disraeli. Šantavý & others (1951) reported that the colchicine contents of the hybrids Lilac Wonder, The Giant and Violet Queen were 0·47, 0·095, 0·113% and the contents of demecolcine were 0·014, 0·014 and 0·067% respectively. For the Disraeli hybrid, we found the content of neutral and phenolic alkaloids of the colchicine-type to agree most closely with that found for the hybrid The Giant but none of the hybrids had a comparable amount of demecolcine. It is therefore possible that both The Giant and Disraeli hybrids have one parent in common which carries the dominant gene for the quantity of colchicine, whereas the parent carrying the dominant gene for the amount of basic alkaloids is different. The low amount of alkaloids in the corm is desirable in a garden plant but this is to some extent off-set by the large amount of alkaloids in the flowers which may prove attractive to children.

C. ritchii. Fahmy (1963) reported that the corms of this species contained only traces of colchicine. The total amount of neutral and phenolic alkaloids, which we have identified as colchicine, colchiceine and Substance C was 0·055% of which 0·050% was colchicine and Substance C. As Substance C was not present in sufficient amounts to react with the spray reagent after chromatography, colchicine must be present in much the greater amount. Thus, this species contains more than traces of colchicine, but not sufficient amounts to make it a valuable source of the alkaloid. Comparatively large amounts of demecolcine were present and the species would be a reasonably good source of this alkaloid.

C. steveni. Šantavý & others (1951) isolated 0·044% colchicine and 0·039% demecolcine from the corms of *C. cilicum* Hayek [= *C. steveni* Kunth (Stefanoff, 1926)]. Our results, from the whole plants, show a

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Footnotes to Table 1.

* All neutral and phenolic alkaloids are expressed as percentage of colchicine and basic alkaloids as percentage of demecolcine. All results are calculated with reference to oven-dried material. The assays for each group were performed on one sample.

** Alkaloids shown present by paper chromatography with solvent system A, distilled water: B, n-butanol-acetic acid-water (4 : 1 : 5).

† number of readings.

‡ Spots of Rf (A) 0·00, 0·63, 0·77; (B) 0·21, 0·75, travelling with solvent front, believed to be some of the non-tropolonic bases reported by Sadykov & Yusupov (1965).

higher concentration of colchicine, 0·068%, but a comparable amount of demecolcine, 0·030%. The plants used by Šantavý & others (1951) were obtained from a bulb merchant and it is probable that they had been under cultivation for several years; the corms we used were cultivated for one year only. Salo (1963) observed that *C. laetum* Stev., when cultivated, yielded less colchicine than in the wild state and it is possible that this is a feature of *C. steveni* also. It is considered doubtful that the presence of the flowers, even if these contain much more colchicine than the flowers of other species examined, would raise the alkaloidal content to 50% more than previously reported results.

C. tunicatum. No work has been published on the alkaloidal content of this species. We found the whole plant to contain large amounts (0·155%) of neutral and phenolic alkaloids of the colchicine type. This is comparable to the colchicine content of *C. autumnale*, 0·15%, obtained by Šantavý (1950). Besides colchicine, *C. tunicatum* contains Substance C, but as this latter gave no colour with the spray reagent on paper chromatograms, it was concluded that it was only present in trace amounts. The results suggest that *C. tunicatum* contains quantities of colchicine sufficient to be a good source of this alkaloid. It also contains comparatively large amounts of demecolcine.

Acknowledgements. We are indebted to Professors J. M. Rowson and G. E. Trease, also Mr. D. Swift, for their helpful criticisms.

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Comparative anatomy of the leaves of *Voacanga schweinfurthii* Stapf and *Voacanga africana* Stapf

F. NEWCOMBE AND B. A. PATEL

There is a marked similarity in the anatomical structures of the leaves of the closely related species *Voacanga schweinfurthii* and *V. africana* which are here described and compared. The evidence suggests that these two species should be considered as a single species.

IN a previous report (Fish & Newcombe, 1966), the taxonomic relationship of the species *Voacanga schweinfurthii* and *Voacanga africana* was discussed. The close similarity of the plants attributed to these species was emphasised with respect to their general morphology and their detailed anatomy, and the chemical constituents of their stem barks. The comparative study has been extended to cover the detailed anatomical structures of the leaves of the two species.

MATERIALS AND METHODS

Two samples of leaves of *V. schweinfurthii* were obtained from Dr. D. B. Fanshawe, Division of Forest Ecology, Kitwe, Zambia; two samples of leaves of *V. africana* were supplied by Dr. M. B. Patel, University of Ife, Nigeria, and a further two supplied by A. G. Kenyon, Tropical Products Institute, London, were also collected in Nigeria. These species were differentiated on the size of the calyx and corolla tube (Pichon, 1947).

Sections were cut with a Reichert freezing microtome. Macerations were prepared using 20% w/v potassium hydroxide or Schultz's solution.

NOTE: The macroscopical and microscopical structures of the leaves of *V. schweinfurthii* and *V. africana* are so similar that a single account is given of their distribution and appearance. Illustrations from prepared sections and macerates are of *V. schweinfurthii*. Similarly, although a full range of measurements was made of the dimensions of the various structures of *both* species, these show such close correlation that only those of one species, *V. schweinfurthii*, are recorded.

Macroscopical characters

The leaves are simple, varying in size from about 6 cm long and 3 cm broad to 17 cm long and 9 cm broad. The shape varies from ovate-lanceolate to lanceolate; the apex is most commonly acute or acuminate but occasionally obtuse; the margin is entire and slightly wavy; the base is symmetrical, cuneate, tapering abruptly to a short or very short petiole. The lamina is greenish-brown to grey on the upper surface which is glabrous except for occasional trichomes on the midrib near the base; the lower surface is greyish-green or pale greyish-brown and varies from glabrous, in older leaves, to smoothly puberulous, in younger ones, the trichomes being most numerous on the midrib and main veins but also

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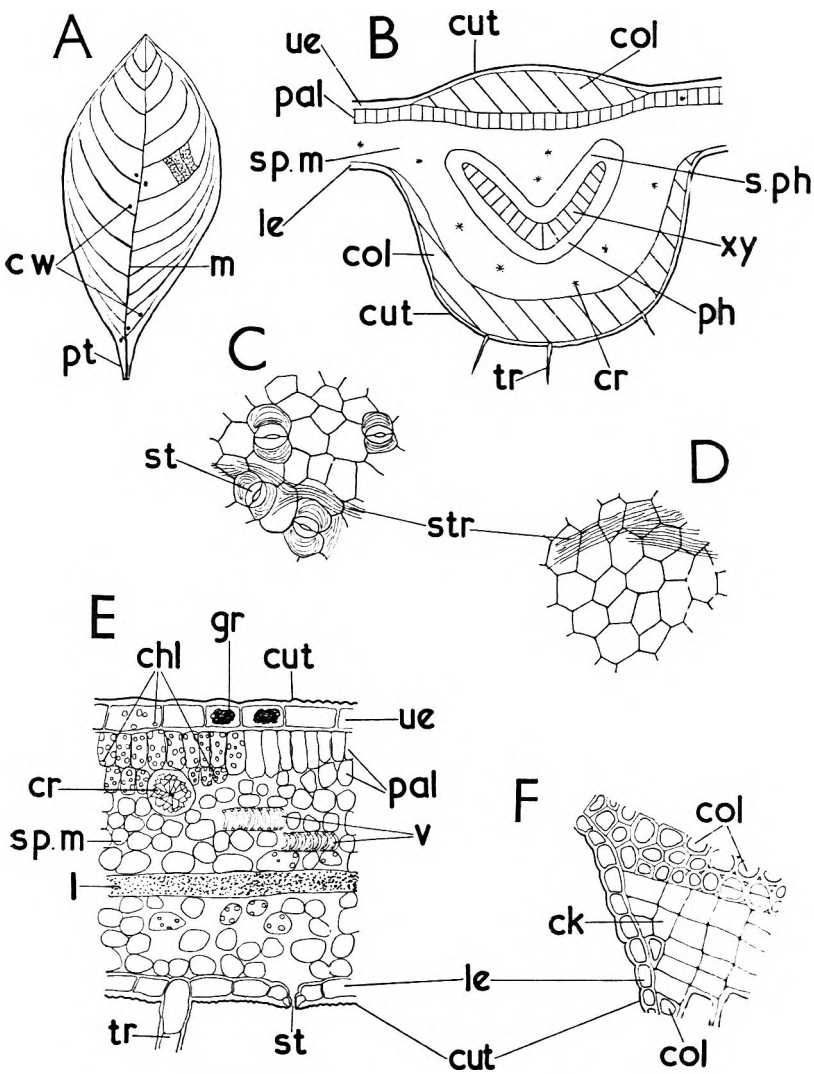

FIG. 1. *Voacanga schweinfurthii* leaf. A, lower surface of leaf. B, transverse section of midrib. C, lower epidermis. D, upper epidermis. E, transverse section of lamina. F, transverse section of cork wart. Magnification: A $\times \frac{1}{4}$, B $\times 20$, C to F $\times 200$. *chl*, chloroplast; *ck*, cork; *col*, collenchyma; *cr*, cluster crystal of calcium oxalate; *cut*, cuticle; *cw*, cork wart; *gr*, granular content; *l*, latex tube; *le*, lower epidermis; *m*, midrib; *pal*, palisade layer; *ph*, phloem; *pt*, petiole; *s.ph*, supernumerary phloem; *sp.m*, spongy mesophyll; *st*, stoma; *str*, striations; *tr*, trichome; *ue*, uper epidermis; *v*, xylem vessel; *xy*, xylem.

being fairly frequent on the interneural areas near the base. Light brown cork warts occur on both surfaces, generally near the base, but most commonly on the lower surface, measuring up to about 2 mm in diameter. The venation is pinnate, about 9 to 19 pairs of lateral veins

leaving the midrib at about 90°, near the base, to 60°, towards the apex, those nearer the apex anastomosing; the midrib and veins are prominent on the under surface and slightly depressed on the upper. The leaves have no characteristic odour, a slightly bitter taste and a brittle and papery texture (Fig. 1, A).

Microscopical characters

LAMINA

The *upper epidermis* consists of a layer of polygonal cells, becoming elongated over the main veins, with straight anticlinal walls, the outer tangential walls being thicker than the inner walls; the cells frequently contain a few chloroplasts or yellowish-brown granular material (Fig. 1, B, D and E). The cells measure about H 16 to **21** to **27** to 33 μ, Lev L 20 to **31** to **35** to 60 μ and Lev B 16 to **20** to **32** to 40 μ. A very few, oval, paracytic *stomata* are present, slightly raised above the epidermis, measuring about 24 μ long and 16 μ broad. The epidermis is covered by a thin *cuticle* exhibiting long, irregular striations which continue over several contiguous cells, being most marked in the areas adjacent to the stomata (Fig. 1, D). Occasional *cork warts* are present consisting of a subcylindrical mass of cells arranged in tabular rows of 3 to 10 cells per row, there being from 10 to 28 rows per wart when seen in either transverse or longitudinal section; small warts are covered by an intact epidermis but larger warts protrude beyond the epidermis, frequently with one or two rows of broken collenchymatous cells on their outer side and a few layers of collenchyma on their inner side. The cell walls are slightly thickened and though always lignified, the intensity of staining with phloroglucinol and hydrochloric acid is very variable; the cells measure about H 10 to 40 μ, Lev L and Lev B 20 to 40 μ (Fig. 1, A and F). *Trichomes* are absent.

The *mesophyll* is well differentiated. The *palisade* consists of two layers of cells; the layer adjacent to the epidermis being of subcylindrical, thin-walled cells measuring about H 13 to **23** to **35** to 39 μ, Lev L and Lev B 7 to **10** to **12** to 17 μ; the cells of the inner layer are somewhat more rounded, measuring about H 16 to **23** to **29** to 33 μ (Fig. 1, B and E). The palisade is continuous over the lateral veins and both layers contain numerous chloroplasts, about 7 to 10 μ in diameter, but no starch. The *spongy mesophyll* consists of about 8 to 12 layers of thin-walled cells, frequently isodiametric but often elongated axially and tangentially, particularly near the lower epidermis, measuring about H 10 to **12** to **17** to 19 μ, Lev L and Lev B 10 to **18** to **34** to 36 μ; the cells are fairly closely packed in places but with numerous large air spaces near the palisade and contain some chloroplasts but no starch (Fig. 1, B and E). Scattered in both the palisade and spongy mesophyll are occasional idioblasts containing cluster crystals of *calcium oxalate*, measuring about 19 to **30** to **41** to 49 μ in diameter (Fig. 1, B and E). Adjacent to the veinlets occurring in the mesophyll are occasional, long, unlignified, cortical *fibres* and occasional, simple *latex tubes*; the veinlets contain *xylem vessels* showing spiral and annular thickening (Fig. 1, B and E).

The *lower epidermis* consists of a layer of polygonal cells elongated over the main veins, with almost straight anticlinal walls; the outer tangential walls are somewhat thickened and covered by a thin *cuticle* having marked, long, irregular striations, particularly prominent near the stomata (Fig. 1, C); the cells have similar contents to those of the upper epidermis and measure about H 6 to **10** to **16** to 19 μ, Lev L and Lev B 16 to **19** to **27** to 33 μ. Numerous, oval, paracytic *stomata* are present, slightly raised above the epidermis, measuring about 24 μ long and 16 μ broad (Fig. 1, C and E). On the interneural regions of the epidermis, there occur occasionally, though sometimes fairly frequently, multicellular, uniseriate, covering *trichomes* with slightly thickened, smooth or very faintly longitudinally striated walls and acute or sub-acute apices; these are of two types, the commoner consisting of 15 to 25 almost isodiametric cells and measuring about 300 to 700 μ in length, the second type consisting of 5 to 12 elongated cells, one or more of which is typically collapsed, measuring about 80 to 700 μ in length. Similar trichomes occur with greater frequency over the veins and midrib (Fig. 2, C) and are accompanied by unicellular, thin-walled, covering trichomes of up to about 30 μ in length, with more or less blunted apices, and well-marked, rounded *papillae* (Fig. 2, B). Glandular trichomes are absent.

MIDRIB

The midrib shows a typical dicotyledonous structure (Fig. 1, B).

The *upper epidermis* is similar to that of the interneural zone, except that the cells are elongated parallel to the midrib, measuring about H 10 to **13** to **16** to 20 μ, Lev L 10 to **16** to **29** to 36 μ and Lev B 7 to **10** to **16** to 20 μ (Fig. 2, A). The epidermis is covered by a fairly thick *cuticle*; *stomata* are absent; *trichomes* are generally absent but when present occur near the base and are of both multicellular, covering types described previously (Fig. 2, C).

The *cortex* contains two prominent zones of very thick-walled, rounded or slightly elongated, collenchymatous cells with few, small, intercellular air-spaces, adjacent to each epidermis, each zone being composed of about 8 to 10 rows of cells near the base, reducing to 2 to 3 rows at the apex. Beneath the upper hypodermal zone of collenchyma is a layer composed of 2 rows of subcylindrical to rounded, thin-walled cells continuous with the palisade of the lamina; this layer is prominent due to the presence in the cells of numerous chloroplasts. The remainder of the cortex is of fairly closely packed, thin-walled parenchyma in which occur elongated, unbranched *latex tubes*, measuring up to 20 μ in diameter, numerous in the region of the pericycle and scattered sparsely towards the outer cortex, with greyish, granular content which stains with Calco Oil Blue, and occasional pericyclic *fibres*, mostly occurring singly but occasionally being in groups of 2 to 4, which are markedly elongated longitudinally with uniformly thickened, unlignified walls and acute or somewhat blunt, unbranched ends; they are about 20 μ thick with lengths of about 2·4 to 3·2 mm. Occasional cluster crystals of *calcium oxalate* are also present (Fig. 1, B; Fig. 2, A; Fig. 3, A).

The *meristele* is arcuate with a central xylem surrounded by phloem (Fig. 1, B). The *phloem* abaxial to the xylem consists of narrow, thin-walled parenchyma; well-marked supernumerary phloem also occurs adaxial to the xylem (Fig. 1, B; Fig. 2, A). Occasional *latex tubes* occur towards the cortical side of the phloem.

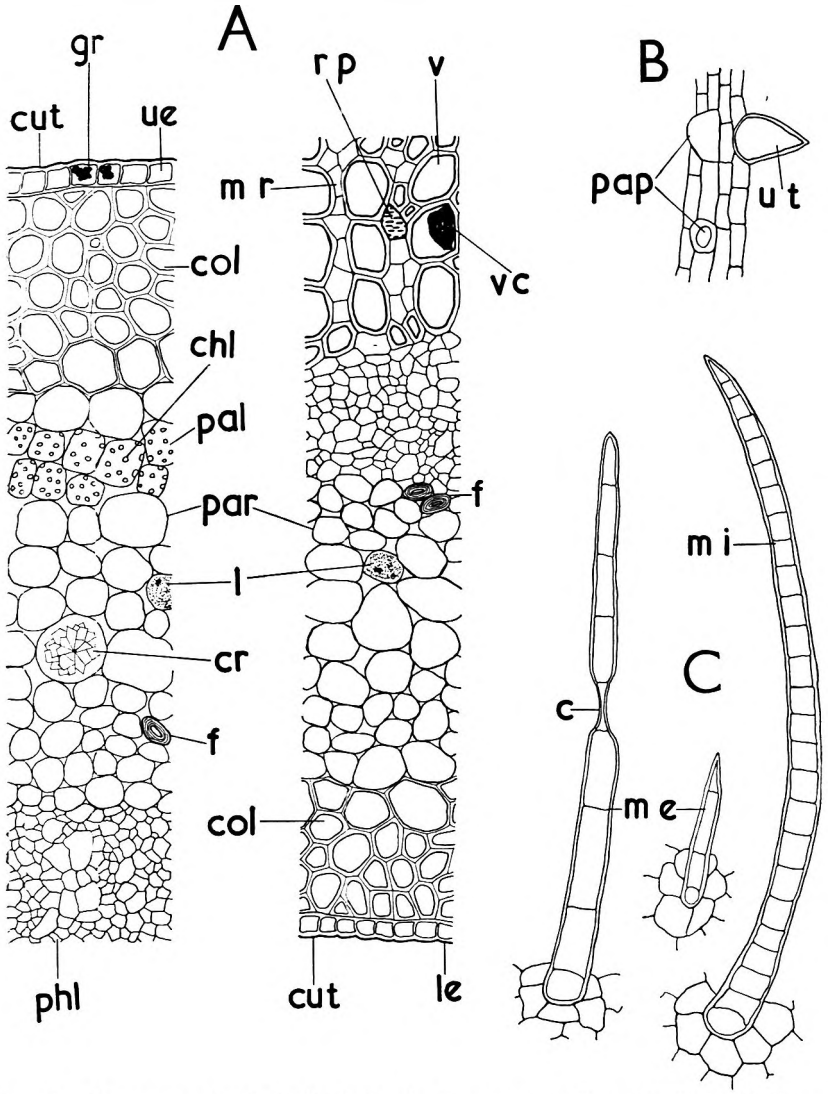

FIG. 2. *Voacanga schweinfurthii* leaf. A, transverse section of midrib. B, lower epidermis of midrib. C, trichomes of midrib. Magnification × 200. *c*, collapsed cell; *chl*, chloroplast; *coi*, collenchyma; *cr*, cluster crystal of calcium oxalate; *cut*, cuticle; *f*, fibre; *gr*, granular content; *l*, latex tube; *le*, lower epidermis; *me*, multicellular trichome with elongated cells; *mi*, multicellular trichome with isodiametric cells; *mr*, medullary ray; *pal*, palisade layer; *pap*, papilla; *par*, parenchyma; *phl*, phloem; *rp*, reticulate xylem parenchyma; *ue*, upper epidermis; *ut*, unicellular trichome; *v*, xylem vessel; *vc*, xylem vessel content.

The *xylem* consists of radially arranged vessels and tracheids exhibiting annular, spiral and reticulate, lignified thickenings, measuring about 10 to 30 μ in diameter, the larger vessels occasionally having some yellowish-brown, amorphous content; and *medullary rays*, one or, less commonly, two cells wide, composed of lignified, reticulate parenchyma, markedly smaller than the conducting elements (Fig. 2, A; Fig. 3, A).

The *lower epidermis* is composed of cells similar to those of the upper epidermis, measuring about H 10 to **11** to **16** to 20 μ, Lev L 16 to **23** to **38** to 40 μ and Lev B 7 to **10** to **14** to 23 μ; the *cuticle* is fairly thick; stomata are absent; *papillae* and both unicellular and multicellular covering *trichomes*, as described previously, occur frequently, though in greatly varying amounts, being most numerous near the base and rare towards the apex (Fig. 2, B and C).

PETIOLE

The *petiole* is subcylindrical with two, slight, longitudinal, lateral ridges and is about 5 to 10 mm long and 2 to 6 mm broad. The structure (Fig. 3, B) is very similar to that of the midrib; the epidermis is of elongated, polygonal cells with very occasional, paracytic *stomata*, numerous *papillae*, frequent unicellular and multicellular covering *trichomes* and covered by by a fairly thick *cuticle*; occasional *cork warts* are present; the hypodermal collenchyma is arranged in a continuous ring; no well-differentiated chlorenchymatous region is present but the cortical parenchyma contains some chloroplasts; a few cluster crystals of *calcium oxalate* are scattered in the cortex; occasional *latex tubes* occur, being most numerous towards the pericycle; occasional pericyclic *fibres* occur; the central arcuate *meristele* is composed of the same type and distribution of cells as in the midrib. Additional structures present are one or more small, round, vascular bundles towards each lateral ridge, with a central *xylem* area of annular and spiral and, very occasionally, reticulate vessels and reticulate, lignified parenchyma surrounded by phloem consisting of narrow sieve tubes with associated parenchyma and occasional *latex tubes* and *fibres* on the periphery (Fig. 3, C).

NUMERICAL VALUES

Determinations of palisade ratio and stomatal index (lower surface) were made. No statistically significant difference between the two species could be shown for these parameters (Table 1).

Using 10 leaves of each species, determinations were made of vein-islet number, veinlet termination number, absolute vein-islet number and absolute veinlet termination number according to the method of Gupta & Kundu (1965) (Table 1). There was no significant difference between the values for absolute vein-islet number. Some difference was suggested by the other parameters but because of the small number of measurements made, further work would be necessary to confirm this. Snedecor's F test and Student's *t*-test were used to assess the measurements made.

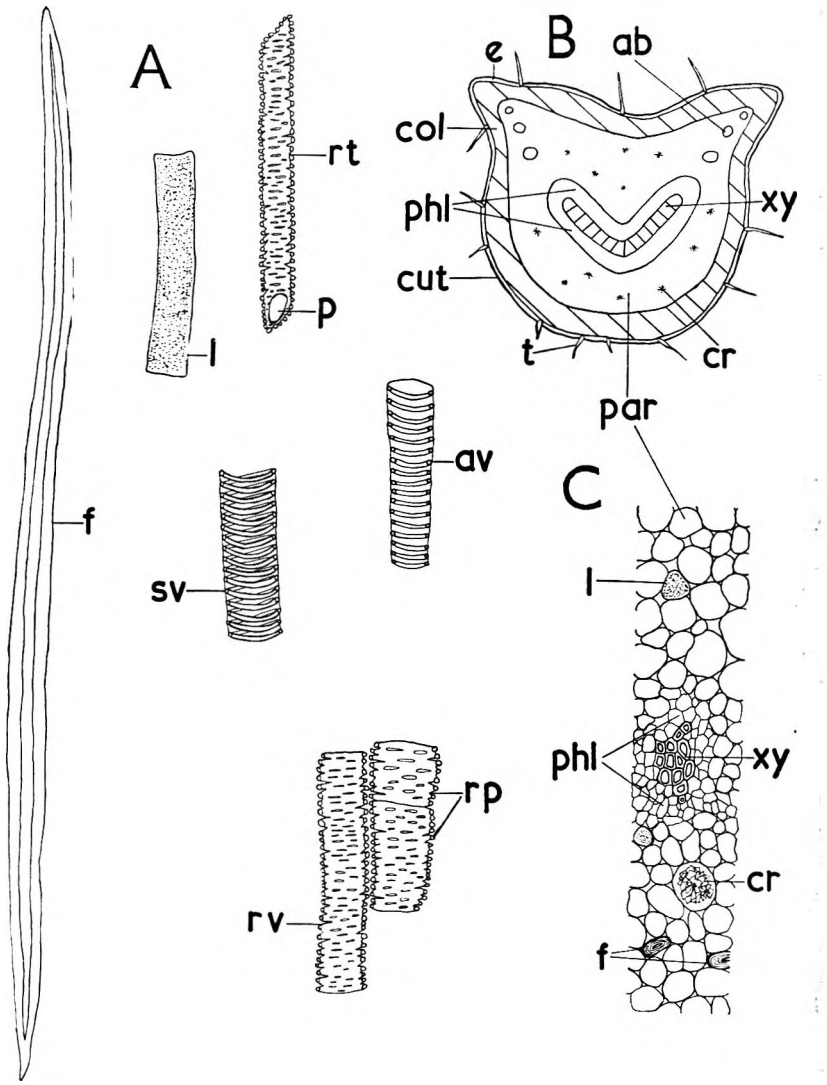

FIG. 3. *Voacanga schweinfurthii* leaf. A, isolated elements of the midrib. B, transverse section of the petiole. C, transverse section of accessory vascular bundle. Magnification: A and C $\times 200$, B $\times 10$. *ab*, accessory vascular bundle; *av*, annular vessel; *col*, collenchyma; *cr*, cluster crystal of calcium oxalate; *cut*, cuticle; *e*, epidermis; *f*, fibre; *l*, latex tube; *p*, pore; *par*, parenchyma; *phl*, phloem; *rp*, reticulate xylem parenchyma; *rt*, reticulate tracheid; *rv*, reticulate vessel; *sv*, spiral vessel; *t*, trichome; *xy*, xylem.

Discussion

Although *V. schweinfurthii* and *V. africana* have always been considered as separate species since their original descriptions (Stapf, 1894,a,b), they can be differentiated (Fish & Newcombe, 1966) on two morphological

characters only. These are the size of the calyx and corolla tube (Pichon, 1947). Both are greater in *V. schweinfurthii*, though there is some overlap of the values for calyx size. Confusion has arisen over the identification of particular plants in these species, several having been placed in one then transferred to the other, and it has been suggested that the differences between the plants of the species may be insufficient to warrant such discrete separation (Newcombe, 1964).

TABLE 1. NUMERICAL VALUES OF *V. schweinfurthii* AND *V. africana*

Character	Species	Range	Variance Ratio (calc.)	V.R. P = 0·05	*t* (calc.)	*t* P = 0·05
Palisade Ratio	*V. schweinfurthii* *V. africana*	3·2– 4·4– 7·4– 9·0 3·5– 4·4– 7·1– 8·5	1·33	1·90	0·43	1·67
Stomatal Index	*V. schweinfurthii* *V. africana*	7·2– 9·8–18·0–26·7 7·4–10·2–17·1–22·2	1·40	1·69	0·28	1·67
Vein-islet number	*V. schweinfurthii* *V. africana*	6·7– 7·5–12·9–14·2 6·0– 7·0– 9·2– 9·5	5·92	3·18	2·27	2·10
Veinlet termination number	*V. schweinfurthii* *V. africana*	14·0–16·5–25·3–27·8 18·0–19·3–23·7–26·3	3·90	3·18	0·37	2·10
Absolute vein-islet number × 10^{-4}	*V. schweinfurthii* *V. africana*	1·1– 1·9– 6·7– 8·8 1·6– 4·1– 8·0– 8·8	1·49	3·18	1·77	2·10
Absolute veinlet termin. number × 10^{-4}	*V. schweinfurthii* *V. africana*	2·1– 3·3–15·1–21·5 3·7– 9·4–18·8–19·4	1·90	3·18	2·31	2·10

The stem barks of the two species have a similar alkaloid content and are structurally indistinguishable (Fish & Newcombe, 1966). Investigation of the macroscopy of the leaves of both species shows that they are indistinguishable, both showing similar variations in size and shape; the type, distribution and sizes of the microscopical structures are also very similar. Leaves of co-generic species are likely to have similar structures but frequently may be differentiated by numerical values. The two most commonly used are palisade ratio and stomatal index, neither of which can be used to differentiate the leaves of *V. schweinfurthii* and *V. africana*.

Although there is a suggestion that the vein-islet number and the absolute veinlet termination number may be different, assessment of the taxonomic significance of these two last-mentioned parameters is difficult since few examples have been reported of absolute veinlet termination number and, regarding vein-islet number, not only may related species have similar vein-islet numbers, e.g. *Barosma betulina* and *B. crenulata*, but also different varieties of a single species may have different vein-islet numbers, e.g. *Barosma serratifolia var. latifolia* and *B. serratifolia* var. *longifolia* (Levin, 1929).

These results support the hypothesis that *V. schweinfurthii* and *V. africana* should be considered as a single species.

Acknowledgement. We wish to thank Dr. K. R. Fell for his interest and guidance in this work.

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Histamine gastric ulceration and its prevention by degraded carrageenan: the effect of aminoguanidine sulphate

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Pretreatment of the guinea-pig with intravenous aminoguanidine sulphate, a drug believed to inhibit histaminase, changes the gastric ulcer response following a dose of histamine acid phosphate (2·5 mg/kg) which elicits a submaximal ulcer response, to that of a dose (5 mg/kg) which normally elicits a maximal gastric ulcer response. A reduction in the volume and acidity of secretion also occurs. Intraduodenal administration of degraded carrageenan prevents this action of aminoguanidine. Conversely, in the presence of aminoguanidine, carrageenan fails to protect against histamine ulceration.

HISTAMINE-induced gastric ulceration can be diminished by intraduodenal degraded carrageenan in the pylorus-ligated guinea-pig (Anderson & Soman, 1963) suggesting a humoral effect of carrageenan. Since it has been shown (Giertz, Hahn, Schmutzler & Seseke, 1964) that the naturally occurring sulphated polysaccharide, heparin, has a histaminase-liberating action in the guinea-pig, we have examined whether histaminase is involved in the ulcer-preventing action of carrageenan. We report results which show that aminoguanidine, a histaminase inhibitor (Schuler, 1952), potentiates histamine gastric ulceration in the guinea-pig and abolishes the ulcer-preventing action of degraded carrageenan.

Experimental

MATERIALS AND METHODS

Seven weight-matched groups of adult male guinea-pigs of a strain susceptible to histamine-induced gastric ulceration (P strain used by Anderson & Soman, 1965, and obtained from Ponchilla Farms, Mapperly, Nottingham) were maintained on diet 18 and fasted 24 hr (water *ad lib.*).

All animals were subjected to identical treatment and operative technique; where a drug was omitted in any group, normal saline (same volume, same route) was administered instead.

Aminoguanidine sulphate. 2 mg/kg intravenously (1 ml/kg in saline) was given at 24 hr and 0 hr before the experiment. In the absence of knowledge of the absorption of aminoguanidine sulphate after administration by other parenteral routes, the intravenous route was used to ensure total availability.

Degraded carrageenan. Ebimar (Glaxo-Evans, Paris) was used (200 mg/ml) (Anderson & Duncan, 1965) in saline; 1 ml solution was given intraduodenally 0·5 hr before, and repeated immediately after, histamine injection.

Gastric ulcer production. The animals were anaesthetised with sodium pentobarbitone, 30 mg/kg i.p., and the gastroduodenal junction ligated.

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After pyloric constriction, histamine acid phosphate (2·5 or 5 mg/kg s.c. in saline, 1 ml/kg) was administered, and degraded carrageenan was injected into the duodenum. The animals were killed 1 hr after the administration of histamine; ulceration was scored on a 4+ scale (Anderson & Soman, 1965) and averaged for each group. In groups 1 and 2 the number of animals showing ulceration was also recorded. Secretion volumes were measured, and free and total acidities titrated using Topfer's reagent and phenolphthalein respectively.

The drugs administered to the various groups are in Table 1.

TABLE 1. EFFECT OF AMINOGUANIDINE SULPHATE ON HISTAMINE ULCERATION IN THE GUINEA-PIG, AND ITS ABOLITION OF THE ANTI-ULCER ACTION OF DEGRADED CARRAGEENAN

Group	No. of animals	Medication	Gastric ulceration	Volume of gastric secretion (ml/kg)	Acidity of gastric secretion (m-equiv./litre)	
					Free	Total
1	12	—	0·13 ± 0·07	14·9 ± 0·9	65 ± 1·4	72 ± 1·3
				P < 0·05	P < 0·001	P < 0·001
2	12	A	0·33 ± 0·09	17·3 ± 0·7	83 ± 2·2	91 ± 2·1
3	16	H_1	2·3 ± 0·35	30·5 ± 1·7	94 ± 4·1	103 ± 3·8
			P < 0·01		P < 0·005	P < 0·005
4	15	A + H_1	3·5 ± 0·32	27·5 ± 1·2	78 ± 2·8	87 ± 2·8
5	8	A + C + H_1	1·9 ± 0·7	38·5 ± 5·2	96 ± 6·3	104 ± 6·2
6	11	H_2	3·63 ± 0·28	26·5 ± 0·8	76 ± 4·1	84 ± 3·6
			P < 0·4			
7	12	A + C + H_2	3·25 ± 0·3	29·7 ± 0·9	78 ± 4·5	86 ± 4·4

Values are averages for the group ± standard error of the mean.
A = aminoguanidine sulphate 2 mg/kg i.v., twice.
H_1 and H_2 = histamine acid phosphate 2·5 and 5·0 mg/kg s.c., respectively.
C = degraded carrageenan 1 ml of 20% solution, intraduodenally, twice.

Results

The results are in Table 1.

The differences in gastric ulceration scores and in the numbers of animals which showed ulceration in groups 1 and 2, were not significant.

Additional experiments were made with two groups of an ulcer-resistant strain, one of 7 and one of 8 guinea-pigs (T strain used by Anderson & Soman, 1965, and obtained from Tuck, Rayleigh, Essex). Animals were treated with aminoguanidine (2 mg/kg i.v., twice) 24 hr before and at the same time as they were given histamine acid phosphate (2·5 mg/kg s.c.). In one group this produced significant ulceration (2·1 ± 0·6); in the other group, degraded carrageenan (2 ml of 20% solution) in addition, failed to reduce this ulceration (1·9 ± 0·7) and to change the volume and acidity of secretion.

Discussion

Aminoguanidine sulphate is believed to inhibit histaminase-type activity (Schuler, 1952; Blaschko, Friedman, Harves & Nilsson, 1959) and Dr. W. Schmutzler tells us that at 2 mg/kg i.v. in the guinea-pig, deactivates plasma histaminase, including that liberated by intravenous sulphated polysaccharide. In the present experiments, reduction of histaminase activity by aminoguanidine could be deduced from the results of group 2

where, although neither the increase in average ulceration nor the number of animals showing ulceration is significantly greater than in group 1, there is a trend towards increased ulceration. Although not significant in itself, this trend should be considered with the simultaneous significant increase in volume and acidity of the gastric secretion. This combined response can be elicited by 0·5–1 mg/kg histamine acid phosphate s.c. in the absence of aminoguanidine. The effect of aminoguanidine may be due either to increased endogenous histamine following inhibition of histaminase, or to the toxicity of aminoguanidine itself, or to both.

The maximum average responses in this type of experiment are 3·0–4·0 (ulcer score), 30–40 ml/kg and 80 m-equiv./litre upwards (volume and acidity of gastric secretion, respectively). Histamine acid phosphate, 2·5 mg/kg s.c., gives a maximum acid response but a sub-maximal ulcer score; 5·0 mg/kg gives a maximum ulcer score but a sub-maximal acid response (Anderson & Soman, 1965). Groups 3 and 6 in the present experiments confirm these findings. When aminoguanidine is given to animals receiving 2·5 mg/kg histamine (group 4) there is a significant increase in gastric ulceration, with decrease in acidity. This change corresponds to the condition obtained by a histamine acid phosphate dose of 5 mg/kg (group 6). These results support the view that pre-treatment with aminoguanidine makes more histamine available *in vivo* and also support the apparent trend to increased ulceration in groups 1 and 2.

The addition of carrageenan (group 5) to the histamine (2·5 mg/kg) and aminoguanidine treatment, restored the response (ulceration and acid) obtained with histamine-aminoguanidine (group 4) to that of group 3 (histamine alone); it appears therefore that dose for dose the histaminase-inhibiting activity of aminoguanidine can be equated to the effect of carrageenan. Dr. Schmutzler further told us that he has found that intravenous degraded carrageenan has a histaminase-liberating effect. The antagonism between aminoguanidine and carrageenan seen in the present experiments is therefore consistent with Schmutzler's observation, and suggests that the effects seen in the stomach after carrageenan given intraduodenally could be due to the histaminase-liberating effect of the carrageenan. Although this implies some uptake of degraded carrageenan by the duodenal mucosa, it does not indicate the origin of histaminase if indeed this is involved.

The results of group 6 show that with a dose of 5 mg/kg of histamine there is a reduction in the volume and acidity of secretion, with an increase in ulceration. Addition of aminoguanidine to this dose of histamine acid phosphate (5 mg/kg) resulted in the death of a number of animals in the group and the results are not included. This was not unexpected since the decrease in acid secretion and the increase in ulceration seen with 5 mg/kg histamine acid phosphate alone is a manifestation of acute histamine toxicity (Anderson & Soman, 1965); addition of aminoguanidine is equivalent to increasing the histamine dosage still further. However, group 7 shows that addition of carrageenan to such a combination maintains all the components of the

response at the level obtained when the 5 mg/kg dose of histamine is given alone (group 6). Conversely, the ulcer preventing action of carrageenan is abolished by aminoguanidine. It is interesting to note that at both doses of histamine, the same doses of aminoguanidine and carrageenan appear to cancel each other out.

It would appear, therefore, that there could be a relation between histaminase and the antisecretory and anti-ulcer activities which have been described (Anderson & Watt, 1959; Bonfils & Lambling, 1960; Anderson & Soman, 1963) for degraded carrageenan.

Two questions remain. Firstly, if we assume uptake of degraded carrageenan by the duodenum, do degraded carrageenan and aminoguanidine interact with mutual inactivation; and secondly, do aminoguanidine and histamine have synergistic toxicity? No evidence for interaction between aminoguanidine and degraded carrageenan could be found *in vitro*; some evidence for synergistic toxicity can be inferred for aminoguanidine and histamine (Schmutzler, 1965).

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Micro-colorimetric determination of cholinesterase activity of motor end plates in the rat diaphragm

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A spectrophotometric method is described for the determination of total cholinesterase activity in groups of 3–30 end plates, after staining with copper thiocholine and dissecting from the rat diaphragm, by continuous recording of the yellow colour (412 mμ) produced by reaction between dithiobisnitrobenzoate (1mM) and thiol groups liberated enzymatically from acetylthiocholine (5mM) at pH 7·0. Cholinesterase activity of end plates after correction for muscle ranged from $3{\cdot}3–22{\cdot}3 \times 10^{-11}$M/end plate/hr and muscle cholinesterase activity ranged from $8{\cdot}8–29{\cdot}4 \times 10^{-11}$ μg/hr. 2·9–26·7% of measured end plate cholinesterase activity was attributable to muscle. The results for end plate cholinesterase agreed with those obtained by other workers using microgasometric techniques. It was calculated that there were approximately $3{\cdot}6 \times 10^6$ cholinesterase active sites/end plate which compared closely with 6×10^6 molecules acetylcholine released/nerve ending/impulse and $2{\cdot}6 \times 10^6$ cholinergic receptors/end plate given in the literature. It is suggested that each acetylcholine molecule after liberation from a nerve ending may interact with one receptor and be destroyed by one cholinesterase active site.

TOTAL cholinesterase activity, that is acetylcholinesterase + butyrylcholinesterase, was determined in groups of end plates dissected from the rat diaphragm which had been stained by a modification of the histochemical procedure for cholinesterase (Koelle & Friedenwald, 1949). Acetylthiocholine was used as a substrate and the thiol groups liberated from this were reacted with copper glycinate to give deposits of copper thiocholine (Malmgren & Sylvén, 1955) on the end plates. This provided a stain adequate for dissection purposes without further development with ammonium sulphide. The groups of end plates taken for cholinesterase determinations contained appreciable amounts of inseparable muscle tissue and therefore the cholinesterase activity of rat diaphragm muscle was also determined.

Experimental

REAGENTS

Staining solution. 5mM acetylthiocholine iodide (Sigma) prepared immediately before use in a mixture containing 5 ml 0·1M glycine, 5 ml 0·02M $CuSO_4$, 5 ml 0·5M $MgCl_2$, and 35 ml pH 6·8 maleate buffer (Gomori, 1955).

Buffer. pH 7·0 phosphate buffer prepared by mixing 28 ml of 0·2M NaH_2PO_4 and 72 ml of 0·2M Na_2HPO_4.

Incubation mixture. 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)/5 mM acetylthiocholine prepared by weighing 0·079 g DTNB (Kodak) and 0·0289 g acetylthiocholine iodide in the same beaker and dissolving immediately before use in 20 ml buffer.

METHOD

Male Wistar rats weighing 120–170 g were decapitated and the diaphragms removed by cutting around the central tendon thus avoiding

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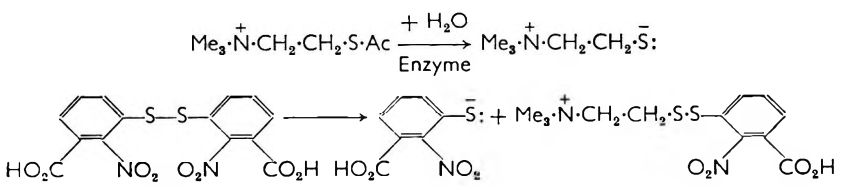
damage to major blood vessels. The diaphragm was then dissected from the ribs and placed in the staining solution for 10 min. The end plates appeared as dots in the characteristic horseshoe arrangement around the centre of the diaphragm. After staining, groups of 3–28 end plates were dissected from the left dorsal tip of the diaphragm under a dissecting microscope at 16× magnification using a scalpel (Swann-Morton, size 15 blade); the diaphragm was held firm by a needle attached to a Singer micromanipulator. Samples of muscle ranging in weight from 2·4–25 μg were similarly dissected from an area immediately adjacent to the end plates and subjected to the procedure as described for end plates.

After dissection the preparation was placed in a Petri dish and the end plates were counted accurately using 16× and 40× magnifications. By means of a glass rod with a tip approximately 0·2 mm in diameter the preparation was then transferred to a cavity slide containing incubation mixture: this rod was used for all manipulations of the preparations. When the preparation became a uniform yellow colour (5–10 min), it was assumed to be saturated with acetylthiocholine and DTNB and for the assay it was transferred to a microcuvette containing 100 μl or 200 μl incubation mixture. The preparation was placed on the meniscus and allowed to come to rest at the bottom of the meniscus before being gently thrust into the solution. This ensured that the preparation was always in approximately the same position at the bottom of the microcuvette and improved the reproducibility of the results.

Two groups of end plates, one muscle sample, and a reagent blank were used in each experiment. The four microcuvettes were placed in a Hilger-Gilford Reaction Kinetics Recording Spectrophotometer with the cell housing maintained at 24–25° and a recording was made over at least 30 min of the change in absorbance at 412 mμ. It was found important to ensure that the light beam in the spectrophotometer passed through the whole height of liquid in each microcuvette. Repeat cholinesterase determinations were made by transferring the preparations to fresh incubation mixture. After estimation each preparation was washed with distilled water on a cavity slide and the water removed by suction. The preparations were then covered with a slide, dried overnight, and subsequently weighed on a quartz fibre fish pole balance (Lowry, 1953).

CALCULATION OF RESULTS

The assay is based on the following reactions (Ellman, Courtney, Andres & Featherstone, 1961):



The enzymatic production of thiol groups from acetylthiocholine was approximately linear during the period of the experiments. Cholinesterase activities were therefore calculated from the slopes of the colour/time curves drawn on the spectrophotometer recordings. The slopes for the reagent blanks were subtracted to correct for non-enzymic hydrolysis. Three determinations were made for each preparation and the mean corrected slope was obtained from the individual corrected slopes: these did not differ by more than 6%. The amount of substrate hydrolysed was calculated as described by Ellman (1959) from the mean corrected slope and the molecular extinction coefficient (ϵ13,600) for the yellow anion produced by the reaction between thiol groups and DTNB. That this coefficient was applicable under the conditions used was checked in control experiments with glutathione. Muscle cholinesterase activity was calculated as moles substrate hydrolysed/μg dry tissue/hr (M/μg/hr) and end plate cholinesterase activity, corrected for muscle, was expressed as moles substrate hydrolysed/end plate/hr (M/ep/hr).

RESULTS

Cholinesterase activities of end plates and muscle were determined in eleven experiments on the diaphragms of different rats and the results are shown in Table 1. In experiments 9, 10 and 11 the reaction volume

TABLE 1. CHOLINESTERASE (ChE) ACTIVITIES OF END PLATES AND MUSCLE IN THE RAT DIAPHRAGM

Expt.	No. of end plates	Weight (μg)	Measured end plate ChE activity (M × 10^{-11}/hr)	Muscle correction (M × 10^{-11}/hr)	Muscle ChE activity (M × 10^{-11}/μg/hr)	Corrected end plate ChE activity (M × 10^{-11}/ep/hr)
1	9	0·4	66·2	6·5	16·2	5·6
	10	0·6	42·6	9·7		3·3
2	12	0·37	133·8	7·6	20·6	10·5
	10	0·95	242·6	19·6		22·3
3	8	0·44	138·2	8·4	19·1	16·2
	6	0·36	58·8	6·9		3·6
4	11	0·98	95·6	8·6	8·8	7·9
	13	0·98	191·0	8·6		14·0
5	11	0·63	148·5	18·5	29·4	11·3
	8	0·67	95·6	19·7		9·5
6	15	0·48	111·7	6·3	13·2	7·0
	14	0·58	130·8	7·7		8·8
7	28	4·18	325·3	43·1	10·3	10·1
	20	5·95	229·3	61·3		8·4
8	12	1·1	164·6	21·0	19·1	1·8
	10	1·4	111·7	26·7		8·5
9	5	0·19	25·7	2·2	11·8	4·7
	8	0·19	76·5	2·2		9·3
10	4	0·4	72·8	4·4	11·0	17·1
	5	0·28	88·2	3·1		17·0
11	5	0·21	62·5	5·9	27·9	1·3
	5	0·30	63·2	8·4		10·9
	3	0·16	38·9	4·5		1·5
				Mean	17·0	10·7
				Standard Deviation	± 6·99	± 4·3

was reduced from 200 to 100 μl which allowed the use of a smaller number of end plates. In experiment 11 three determinations were made alternately on three groups of end plates and three samples of muscle.

The Table shows that after correction for muscle the cholinesterase activity of end plates ranged from 3·3–22·3 $\times$ 10^{-11}M/ep/hr and the cholinesterase activity of muscle itself ranged from 8·8–29·4 $\times$ 10^{-11}M/μg/hr. When activities were expressed on a weight basis the proportion of the measured end plate cholinesterase activity attributable to muscle ranged from 2·9 to 26·7% (mean 11%). There was no relationship between the corrected end plate cholinesterase activity (M/ep/hr) and muscle cholinesterase activity (M/μg/hr).

Discussion

The results obtained for end plate cholinesterase activity using the described method agree closely with the published data from microgasometric work when the latter were converted from μl CO_2 produced to moles substrate hydrolysed/end plate/hr. Thus the range of 9·9–28·6 $\times$ 10^{-11}M/ep/hr (Giacobini & Holmstedt, 1960) for the total cholinesterase activity of end plates from the rat rectus abdominis, and the corresponding values of 14·3–20·1 $\times$ 10^{-11}M/ep/hr and 22·3–53·6 $\times$ 10^{-11}M/ep/hr for the mouse diaphragm and gastrocnemius respectively (Brzin & Zajicek, 1958) compare well with the range of results reported in this paper for the rat diaphragm (3·3–22·3 $\times$ 10^{-11}M/ep/hr). It is interesting to note that Giacobini & Holmstedt (1960) gave results for unstained end plates since these could be visualised relatively easily in the rat rectus abdominis. On the other hand the results given by Brzin & Zajicek (1958) were obtained with end plates which had been stained by a similar method to that described for the rat diaphragm. It would therefore appear that the staining solution does not markedly affect the activity of the enzyme.

It is not known to what extent muscle cholinesterase contributed to the above results for endplate cholinesterase activity obtained by Giacobini & Holmstedt (1960) and Brzin & Zajicek (1958) although the results of our experiments indicate that muscle may contribute up to approximately 27% of the measured end plate activity. Corrected cholinesterase activities were expressed per end plate rather than on a weight basis since the weights of groups of end plates assayed were assumed to be due mainly to muscle. The sizes of end plates varied from 13·0–35·1 μ (length) and 6·5–22·1 μ (breadth) but no relation was observed between the dimensions and the cholinesterase activities of the end plates. However the complexity of end plate morphology may not permit calculation of the enzyme concentration per unit of surface area or volume since the anatomical structure resembles a tree more than a plate (Giacobini & Holmstedt, 1960).

The cholinesterase activity of muscle has been studied previously in some detail using short segments of single muscle fibres by means of a sensitive microgasometric technique (Giacobini & Holmstedt, 1960). Results which were given per unit volume of muscle cannot be compared

accurately with the results given in this paper for relatively large samples of tissue which may contain varying amounts of connective tissue and blood. A rough comparison is possible however between the cholinesterase activities of muscle ($8{\cdot}8$–$29{\cdot}4 \times 10^{-11}$M/$\mu$g/hr) and myosin (18 μg acetylcholine/mg protein/hr; 10×10^{-11}M/μg protein/hr) (Varga, König, Kiss, Kovacs & Hegedüs, 1955) and it is therefore possible that myosin may contribute appreciably to the cholinesterase activity of muscle.

The mean result obtained for end plate cholinesterase activity is $10{\cdot}7 \times 10^{-11}$M/ep/hr and the number of cholinesterase active sites per end plate can be calculated approximately by dividing the end plate cholinesterase activity expressed as $10{\cdot}7 \times 10^{11}$ acetylcholine molecules/ep/min by the turnover number of $2{\cdot}95 \times 10^{5}$ acetylcholine molecules hydrolysed/min/acetylcholinesterase active site (Cohen & Warringa, 1953). The value obtained, $3{\cdot}6 \times 10^{6}$ cholinesterase active sites per end plate, compares closely with 6×10^{6} acetylcholine molecules liberated/nerve ending/impulse obtained from the results given by Krnjević & Mitchell (1961), and also with the number of cholinergic receptors ($2{\cdot}6 \times 10^{6}$)/end plate determined by Waser (1962). Although these results have been obtained under widely different conditions they suggest that there are approximately the same number of receptors and cholinesterase active sites as the number of acetylcholine molecules released per nerve ending per impulse. If correct it may be assumed that after liberation from a nerve ending each molecule of acetylcholine interacts with one receptor and is destroyed by one cholinesterase active site.

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The influence of the female sex hormones on the response of the pig uterus to electrical stimulation

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The response of the pig myometrium to electrical stimulation *in vitro* was used to assess the influence of the female sex hormones at different stages of the oestrus cycle. Optimal stimulation produced greater tension in the mature uterus than in the immature uterus and the tension was greater in the oestrogen-dominated uterus than in the progesterone-dominated uterus. The minimum (threshold) voltage necessary to elicit an isometric contraction was not influenced by the stage of the uterus in the oestrus cycle. Varying the frequency of stimulation caused changes in tension indicating the predominant sex hormone. The progesterone-dominated myometrium appears to bind calcium more strongly than the oestrogen-dominated myometrium.

IN many species spontaneous uterine motility is of different types according to the stage of the oestrus cycle. This was recognised by Corner (1923) who correlated the period of suppressed uterine motility with the presence of corpora lutea in the ovaries. The respective influence of oestrogens and progesterone on the myometrium has been studied using as a criterion the response of the uterus to electrical stimulation. Thus Csapo & Corner (1952) found in the rabbit uterus, that when the frequency of electrical stimulation was varied, the tension developed was proportional to the frequency of stimulation (positive staircase) in oestrogen-dominated uteri, and inversely proportional (negative staircase) in progesterone-dominated uteri.

The oestrus cycle in the pig is of three weeks' duration and has been described in detail by Burger (1952). Oestrus lasts for two or three days, ovulation usually occurring on the second day of oestrus. Corpora lutea reach maximum size in the second week of the cycle and after the 14th day decrease with the approach of oestrus. During this period there is a rapid growth of those follicles destined to rupture at the next oestrus period.

The work described in this paper is an *in vitro* study of the influence of the female sex hormones, revealed by electrical stimulation, on the myometrium of a polyoestrous animal, the pig. Non-pregnant uteri at different stages of the oestrus cycle were used to avoid the complications of hypertrophy and stretching of the uterus which occur during pregnancy.

Experimental

Collection of material. Uteri were collected from a nearby abattoir from freshly slaughtered Large White pigs; strips of uterus were set up in the tissue baths within 25 min of the animal being stunned. Immediately the uterus was removed from the animal it was examined to ascertain that it was non-gravid and that the ovaries appeared to be active. The anterior (ovarian) half of the right horn was removed and transferred to a thermos flask containing modified Krebs solution which had previously

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been cooled to 4° and gassed for 5 min with a mixture of 95% oxygen and 5% carbon dioxide. The modified Krebs solution was made up as follows (conc. g/100 ml): NaCl 0·69, KCl 0·035, $CaCl_2$ 0·028, $MgCl_2$ 0·011, $NaHCO_3$ 0·21, NaH_2PO_4 0·014, glucose 0·2. The weights refer to anhydrous salts.

Setting up the tissues. A longitudinal strip 3 cm long and 7 mm wide was cut from the antimesometrial border at the severed end of the uterine horn. The endometrium was removed and the strip of myometrium attached to a hook of platinum wire fixed to a Perspex frame in a 10 ml tissue bath; the hook also served as stimulating electrode. The upper end of the strip was connected by a thread to an isometric lever. A piece of silver wire inserted in the upper end of the strip acted as the second electrode. Krebs solution at 39° and gassed with 95% oxygen and 5% carbon dioxide was used in the bath.

The lever consisted of a watch spring, the tension on which could be adjusted to allow the uterine strip a maximum shortening of less than 5% of the resting length. A light lever arm was attached to the centre of the spring so as to give a 25 × magnification of the contractions which were recorded on a smoked drum.

For 10 min after being set up in the tissue bath, the strip elongated. At 2 min intervals during this period, the tissue was stretched slightly by lowering the Perspex strip in the tissue bath and the resting length was determined by the last stretch to which the uterus adjusted without developing resting tension (Csapo, 1954). The Perspex strip was marked at 2 mm intervals with the platinum hook as zero, and the resting length of the tissue was noted when stretching was completed; all the muscle strips used had a resting length of 32–38 mm.

Assessment of the stage in the oestrus cycle. The numbers of follicles or corpora lutea in each ovary were recorded. The stage of each uterus in the oestrus cycle was then determined as described by Corner (1921).

Threshold voltage and optimum voltage. After adjusting the strip to its resting length it was stimulated electrically at intervals of 1 min, each impulse of 5 sec duration, increasing the intensity of successive stimuli by increments of 1 V. When the muscle first contracted, the voltage which induced the contraction (threshold voltage) was noted, and also the voltage which induced maximal tension (optimum voltage). Voltage was then decreased with successive stimuli and the threshold voltage again determined. The strip was rested for 15 min.

The staircase effect was determined according to the method described by Schofield (1954). If the tension in the strip decreased when the frequency of stimulation was reduced, the effect was called a positive staircase, whereas if the tension increased with reduction in the frequency of stimulation, the staircase was negative. A transient staircase was designated as one in which there was no alteration in tension at different frequencies of stimulation.

Effect of calcium depletion on electrically induced tension. After recording the staircase effect, the tissue was rested for 15 min and then stimulated at the previously determined optimum voltage, each stimulus of 5 sec

duration at a frequency of 1/min until a steady state tension was established. Calcium-free Krebs solution was then substituted for the usual Krebs solution and this point of the experiment designated as zero time. Thereafter, at alternate 1 min intervals, the strips were stimulated at optimum voltage and the solution in the baths changed; recordings were continued for 1 hr from zero time.

Effects of progesterone. The effects of water-soluble progesterone (Intravenous Primolut, Schering) on spontaneous and electrically-induced uterine contractions were investigated using uteri in the oestrus stage of the cycle. After adjusting the strip to its resting length and determining the optimum voltage for stimulation, a period of 1½ hr was allowed to elapse for the spontaneous contractions to become regular. Progesterone was diluted in Krebs solution to give final concentrations of 20 40, 60 and 80 μg/ml.

The bath was emptied and then filled with 20 μg/ml progesterone solution previously warmed to 39°; the solution remained in the bath until the maximum effect was observed before it was replaced by fresh Krebs solution. When spontaneous contractions became regular again, the experiment was repeated using 40 and 60 μg/ml progesterone.

After a resting period of 30 min, the strip was stimulated at a frequency of 1/min, each stimulus being of 5 sec duration at the previously determined optimum voltage; this was continued throughout the remainder of the experiment. When a steady state tension was established, the Krebs solution was replaced by successively greater concentrations of progesterone as described above.

Results

The staircase effect. The staircase effect, illustrated in Fig. 1, was studied in 64 non-pregnant sow uteri at different stages of the cycle and the results are shown in Table 1.

TABLE 1. THE TYPES OF STAIRCASE RECORDED FROM UTERI AT DIFFERENT STAGES OF THE OESTRUS CYCLE. The stage of the cycle was assessed on histological evidence and the figures in each group are the numbers of uteri from which the different staircases (positive, negative or transient) were recorded.

Type of staircase	Stage of the oestrus cycle			
	Oestrus	4–7 days	8–14 days	15–20 days
Positive	0	11	2	4
Negative	0	3	28	0
Transient	8	3	3	2

Negative staircases were recorded from those uteri in which the ovaries contained mature corpora lutea. The 5 specimens in the group in the 8–14 days stage of the cycle which did not show negative staircases were not typical of the group, since the ovaries contained large follicles in addition to corpora lutea. Blood levels of progesterone in sows during this stage of the oestrus cycle are comparable to the levels during pregnancy (Short,

1957, 1960; Rowlands & Short, 1959). It is therefore concluded that a negative staircase indicates a progesterone-dominated myometrium.

Histological examination of the corpora lutea of those uteri in the 4–7 days stage of the cycle revealed all stages of development from early post-oestrus, with a large blood clot in the centre, to practically fully formed corpora lutea. The 11 uteri in this group from which positive staircases were recorded were in the early post-oestrus stage of the cycle (3 or 4 days from the beginning of oestrus) in contrast to the remaining 6, the ovaries in which contained more mature corpora lutea. It has been shown that in the sow, oestrogen levels in the urine (Velle, 1960; Raeside, 1961), and in the ovaries (Allen & Doisy, 1927) are highest during oestrus. Thus it is concluded that a positive staircase indicates an oestrogen-dominated myometrium.

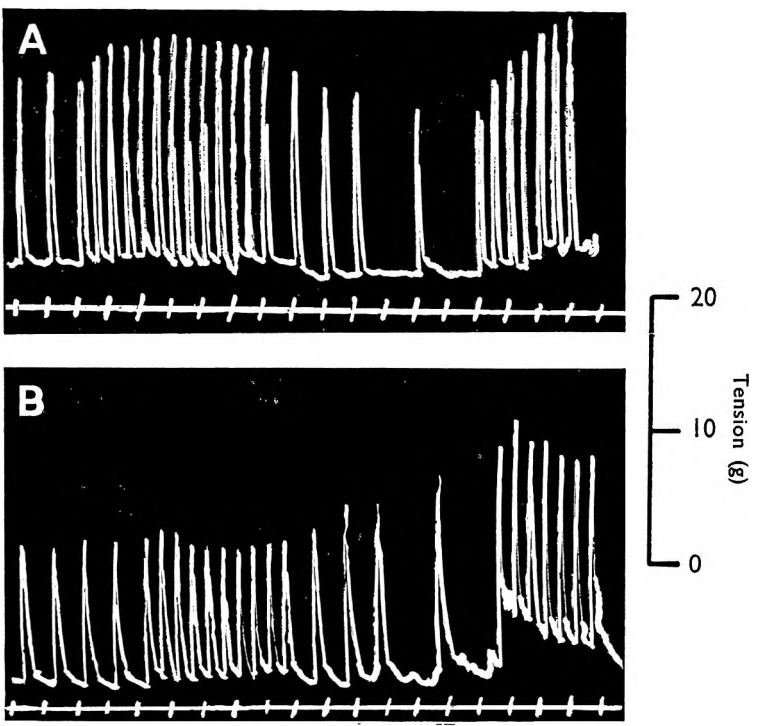

FIG. 1. A. A positive staircase recorded from a strip, the ovaries of which contained newly-ruptured follicles. The tension developed is proportional to the frequency of stimulation. B. A negative staircase recorded from a strip in the 8–14 days stage of the oestrus cycle; the ovaries contained mature corpora lutea. The tension developed is inversely proportional to the frequency of stimulation. Time marker 1 min.

The anomalous result that transient, rather than positive, staircases were recorded from uteri whose ovaries contained mature, unruptured follicles does not necessarily detract from the inferences already drawn. Spontaneous uterine motility at this stage of the cycle is characterised by

contractions of very regular amplitude and frequency (Keye, 1923; Wislocki & Guttmacher, 1924; King, 1927; Adams, 1940). Furthermore, in such strips oxytocin does not cause a significant increase in amplitude of contractions, but produces either an increase in the frequency of contractions, or a more sustained contraction (Adams, 1940). It is not unexpected, therefore, that strips from uteri in the oestrus stage of the cycle respond with maximum tension to optimal electrical stimulation.

Some of the uteri whose ovaries contain either developing corpora lutea (4–7 days stage), or degenerating corpora lutea with developing follicles (15–20 days stage), would be expected, from the evidence cited above, to be neither oestrogen-dominated nor progesterone-dominated; that transient staircases were recorded from such uteri is in accord with this argument.

The staircase effect was also studied on 16 immature gilt uteri. In all instances, electrical stimulation caused only small responses with no change in tension when the frequency of stimulation was altered.

Threshold voltage. The minimum (threshold) voltage causing an isometric contraction was measured in each strip after adjusting the resting length; it was expressed as V/cm of the resting length. The uteri were subsequently divided into 2 groups on the basis of histological examination and the type of staircase. One group consisted of 24 uteri in the oestrus (transient staircases) and the early post-oestrus stage of the cycle (positive staircases); that is, the myometrium was oestrogen-dominated. The second group consisted of 25 uteri in the 8–14 days stage of the cycle (negative staircases) with a progesterone-dominated myometrium.

A statistical analysis of the mean values for threshold voltage shows no significant difference between the 2 groups.

Attempts were also made to measure threshold voltages in strips from immature gilt uteri. However, by the time the strips were adjusted to the resting length there was marked spontaneous motility and it was impossible to determine with accuracy whether the smaller voltages induced contractions or whether the contractions were spontaneous.

Steady state tensions. Before studying the response to varying the frequency of electrical stimulation (staircase effect), the strips were stimulated at optimum voltage at a frequency of 1/min until a steady state tension was established and this value was measured for each strip. The sow uteri were subsequently divided into the same 2 groups as previously described; strips from immature gilt uteri were also examined and the results are summarised in Table 2.

TABLE 2. THE MEAN VALUES FOR STEADY STATE TENSION IN A GROUP OF IMMATURE GILT UTERI AND 2 GROUPS OF SOW UTERI AT DIFFERENT STAGES OF THE OESTRUS CYCLE

	Sows	
Immature gilts	Oestrogen-dominated	Progesterone-dominated
Tension (g) ± s.e. 7·6 ± 0·55 (16)	Tension (g) ± s.e. 16·7 ± 1·14 (24)	Tension (g) ± s.e. 11·1 ± 0·62 (25)

No. of uteri in parentheses

The mean tension developed in the gilt uteri is significantly less ($P < 0{\cdot}01$) than that in the sow uteri. Furthermore, a comparison of the 2 groups of sow uteri shows that the mean tension in the progesterone-dominated group is significantly less ($P < 0{\cdot}01$) than that in the oestrogen-dominated group.

Effect of calcium depletion on tension. The effect of repeatedly rinsing strips in calcium-free Krebs solution was studied in 29 uteri, 8 of which were from immature gilts and 21 from sows. On the basis of histological evidence and the staircase effect, the sow uteri were classified into 10 which were oestrogen-dominated and 11 which were progesterone-dominated.

The results are summarized in Fig. 2, which shows that tension was reduced most rapidly in the oestrogen-dominated strips. A statistical comparison of the 2 groups of sow uteri was made by determining graphically the time at which the electrically induced tension in each strip was reduced to 50% of the steady state tension. The difference between the 2 groups is highly significant ($P < 0{\cdot}01$).

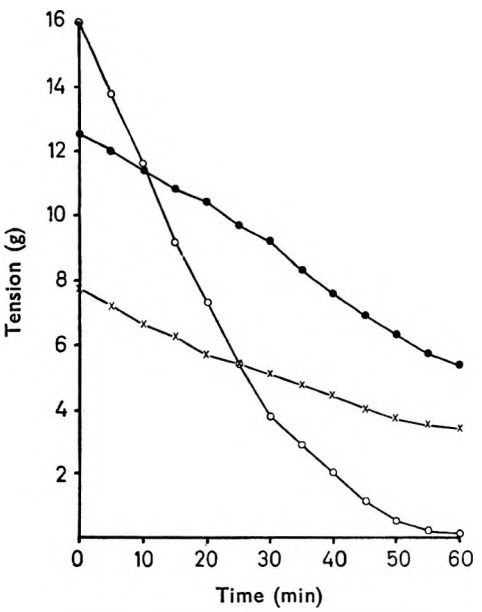

FIG. 2. The rate of reduction of electrically-induced tension in calcium free Krebs solution in strips at different stages of the oestrus cycle. The open circles represent the mean tensions of 10 oestrogen-dominated strips. The filled circles and the crosses represent respectively the mean results from 11 progesterone-dominated strips and 8 strips from immature gilt uteri. At zero time, calcium-free Krebs solution was substituted for normal Krebs solution.

Effects of progesterone. In these experiments, 6 uteri in the oestrus stage of the cycle were used; during this stage spontaneous motility is pronounced and regular, thus any effects of progesterone could be unequivocably demonstrated, and furthermore could be accurately

expressed as a percentage reduction in tension. In all cases spontaneous motility was completely abolished by 60 μg progesterone/ml. Progesterone also reduced the tension of electrically induced contractions, and Fig. 3 shows that there is a satisfactory correlation between dose and response.

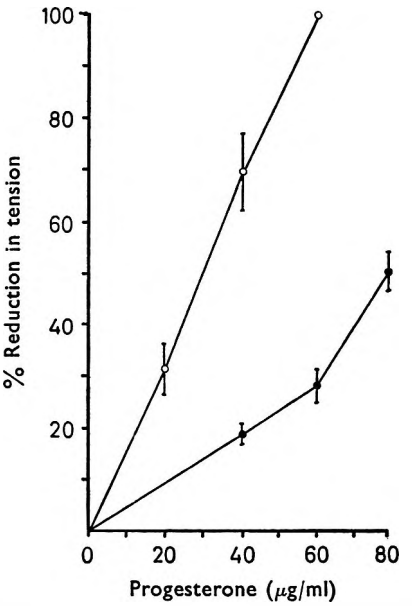

FIG. 3. The reduction in tension produced by progesterone on spontaneous contractions (open circles) and electrically-induced contractions (filled circles). Each point represents the mean of 6 experiments, and the bars indicate $\pm$s.e. of the mean.

Discussion

This investigation has shown that the type of staircase recorded from the pig myometrium is correlated with the stage of the oestrus cycle. The evidence for the staircase effect in the pig uterus is circumstantial, for final proof would necessitate ovariectomising animals and treating some with oestrogen and others with progesterone before recording staircases. Nevertheless, it is concluded that the staircase effect (with the exception of the uteri in oestrus) is similar to that described in the rabbit (Csapo & Corner, 1952; Schofield, 1954) and sheep (Bengtsson & Schofield, 1960).

The threshold voltage required to produce an isometric contraction in the oestrogen-dominated pig uterus is not significantly different from that in the progesterone-dominated uterus. This is contrary to the finding of Csapo & Goodall (1954) that in the rabbit, the progesterone-dominated uterus has a significantly higher threshold for electrical stimulation than the oestrogen-dominated uterus. Their results for the rabbit are in accord with the observations that progesterone influences the myometrium by raising the membrane potential to a higher value than that in the oestrogen-dominated myometrium (Goto & Csapo, 1959; Kuriyama & Csapo, 1961;

Marshall & Csapo, 1961). The evidence that the membrane potential in the progesterone-dominated myometrium is greater than that in the oestrogen-dominated myometrium is not however, unequivocal, for in the rat, Jung (1964) found that there was no difference in membrane potential between oestrogen-dominated and progesterone-dominated uteri. The results of the present study are more readily explicable in the light of Jung's (1964) observations.

Csapo (1950) demonstrated that the synthesis of actomyosin in the myometrium is directly related to the influence of oestrogen and is not affected by progesterone. Further, it has also been shown (Csapo & Corner, 1953) that, other factors being equal, the tension developed by the myometrium to optimal electrical stimulation is proportional to the actomyosin concentration. It is concluded, therefore, that the greater tension produced by electrical stimulation of the sow myometrium when compared with the gilt, is due to a higher concentration of contractile protein in the former, produced by the influence of oestrogen. Unpublished observations by Csapo (cited by Reynolds, 1951), in which actomyosin concentration in the sow myometrium was measured directly, revealed no differences in concentration at different stages of the oestrus cycle. It is therefore concluded that the tension induced by electrical stimulation in the progesterone-dominated myometrium is less than that in the oestrogen-dominated myometrium, not because of any difference in actomyosin concentration, but because of the effect of progesterone. This conclusion is further substantiated by the experiments in which progesterone was added to tissue baths containing strips from uteri in oestrus. There was a reduction both in the tension developed during spontaneous contraction and in the tension developed to optimal electrical stimulation, with a correlation between dose and response. It has also been demonstrated that progesterone depresses uterine motility *in vitro* in the rabbit (Kuriyama & Csapo, 1959), rat, human (Jung, 1964) and the guinea-pig (Sullivan, 1963). The finding that the mean steady state tension in the progesterone-dominated group of sow uteri is less than the mean steady state tension in the oestrogen dominated group, despite similar actomyosin concentrations in each, is in agreement with conclusions drawn by Schofield (1955) from *in vivo* studies in rabbit uteri.

The role of calcium in the response of smooth muscle to drugs has been discussed by Daniel (1964). The present work has shown that the progesterone-dominated sow myometrium binds calcium more strongly than the oestrogen-dominated myometrium; similar findings have been reported in rabbits (Csapo, 1956; 1961), but preliminary studies in the guinea-pig (Schofield, 1964) have not shown this effect. Although the evidence as yet is insufficient to draw firm conclusions, the different effects of the female sex hormones on calcium binding in the myometrium may help to explain the relative refractoriness of the progesterone-dominated uterus to stimulation.

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The compaction properties of sodium chloride in the presence of moisture

E. SHOTTON AND J. E. REES

The effect of moisture on the behaviour of sodium chloride under compression was investigated by measurement of compaction forces and ejection forces. Saturated solution present at the crystal surfaces effected a reduction in friction at the interparticulate boundary and between the particles and the die wall. Dry material formed compacts of higher strength than wet material except at low pressures. Subsequent drying of compacts prepared in the presence of moisture resulted in an increase in strength due to interparticulate recrystallisation.

MANY of the difficulties encountered during the preparation of compressed tablets may be attributed to an unsuitable moisture content. The effects of moisture on the compaction properties of peat (Matveev, 1951; Naumovich, 1957), coal (Crone & McKee, 1950), soil (Maclean, 1948; Lewis, 1959) and brick (Boyd, 1949) have been reported. The present work was performed to investigate the influence of moisture on the behaviour of a soluble particulate solid during compression, and on the properties of the compact produced. Jaffe & Foss (1959) state that removal of water of crystallisation prevents the formation of tablets from materials which normally bond by direct compression. Other reports (Ferrand, 1955; Martin & Cook, 1961) have quoted values of relative humidity at which the preparation of compressed tablets is facilitated. Discrepancies between these values suggest that optimum ambient conditions are specific to individual materials. Seth & Münzel (1959) and Egorova & Vikul'eva (1961) derived an optimum granular moisture content which was specific to a given granulation and yielded tablets of greater strength. At higher moisture content adhesion of material to the punch faces occurred whereas, in certain cases, a low moisture content resulted in "capping" and lamination of the tablets.

In the present investigation it was considered advisable to avoid additives and sodium chloride was consequently selected as an ionic, cubic crystalline material, capable of being compressed directly to form a coherent compact.

Experimental

A Lehman single punch eccentric tablet machine was instrumented with Saunders Roe $\frac{1}{8}$ inch linear foil resistance strain gauges (60 ohm nominal resistance, gauge factor 2·07) in a manner similar to that described by Shotton & Ganderton (1960). Two gauges were bonded to the 1·2 cm diameter plane-faced upper punch (K9 tool steel, surface ground to 5 microinches) using Eastman 910 cement, and three gauges were bonded to the lower punch holder. The gauges and terminals were then coated with a polysulphide rubber compound. The technique of Shotton & Ganderton (1960) was employed to calibrate the strain gauge circuits. The

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machine was installed in a room in which the ambient relative humidity was controlled.

The weighing assembly of a Cahn Gram Electrobalance was installed in a vacuum oven and employed for thermogravimetric determination of moisture. A loss in weight of 0·05 mg could be detected in a sample weight of 900 mg (equivalent to approximately 0·005% moisture).

A batch of sodium chloride (B.P. quality) was screened to obtain a 30–40 mesh fraction. This fraction was then subjected to further separation on a Lavino Alpine Air-Jet Sieve to remove fines of −100 mesh size adhering to the surface of the larger crystals.

Weighed samples of the screened sodium chloride, sufficient to produce a compact of 0·4 cm thickness at zero porosity in a 1·2 cm diameter die, were stored in glass vials contained in a constant humidity chamber. The time of storage depended on the number of samples present and was varied to produce a range of moisture contents. When this had reached the required value the containers were removed from the humidity chamber and rapidly sealed. Preliminary experiments with the Cahn balance had indicated a high rate of moisture loss from the surface of cubic crystals at normal ambient conditions. Consequently, the conditions in the room were maintained identical to those in the humidity chamber.

Six humidified samples were compressed at each selected pressure and measurements of upper and lower punch forces and ejection forces were recorded. The resulting compacts were weighed and the dimensions of each were measured. Three compacts at each pressure level were tested using a diametral crushing apparatus (Shotton & Ganderton, 1960). The three remaining compacts were similarly examined after drying to constant weight over silica gel.

A binocular microscope was employed to examine the fractured surfaces of the compacts.

The compressional behaviour of sodium chloride, previously dried at 110° and in which no moisture could be detected, was investigated by two methods. In one experiment, the die wall was "conditioned" by compaction of two samples of dry material before measurements were made of compression forces on subsequent samples. The die was cleaned before compression of samples at a different pressure. In the second experiment the die was cleaned before the preparation of every compact.

Results

Compression

The effect of moisture on the proportion of force transmitted to the lower punch through the mass is shown in Fig. 1, where R is the ratio of maximum transmitted force, F_B, to maximum applied force, F_A, during a compression cycle. A low moisture content (0·02% w/w) produced a large increase in F_B at low applied force compared with anhydrous sodium chloride compressed in a conditioned die. As the applied force increased, the punch force ratio decreased.

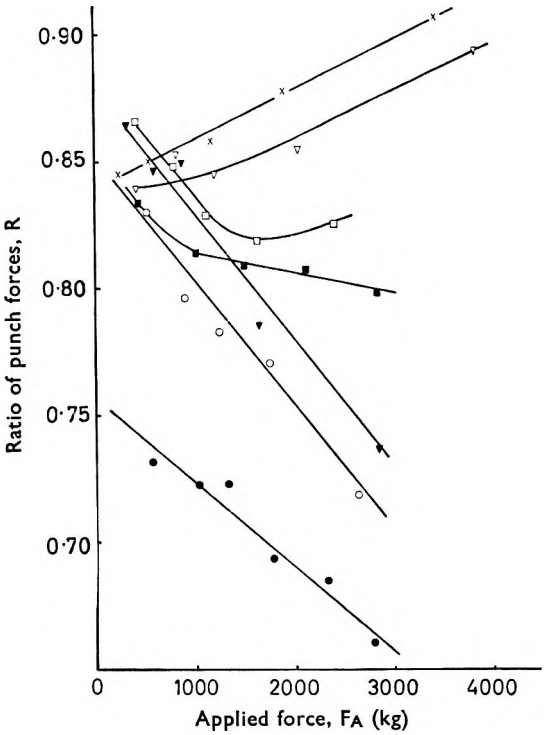

FIG. 1. Effect of moisture content on the ratio of transmitted punch force to applied punch force. Moisture content %: ×, 10; ▽, 2·4; □, 0·55; ■, 0·16; ○, 0·02; ▼, 0 (clean die); ●, 0 (conditioned die).

Force lost to the die wall, F_D, due to frictional resistance is equivalent to the difference in the values of applied and transmitted force, and variation of this parameter with increasing applied force is recorded in Fig. 2. For 0·55% moisture, the relationship was linear up to 1600 kg applied force (Fig. 2); above this the slope of the graph decreased and the punch force ratio, R, increased (Fig. 1). In the presence of 2·4 and 10% moisture, the values of R at low applied force were lower than for 0·55% moisture, and a continual increase in R occurred with increasing applied force. At high values of applied force, an increase in moisture content resulted in an increase in transmitted force.

EJECTION

Anhydrous sodium chloride compressed in a conditioned die exhibited a linear relationship between ejection force and applied force (Fig. 3). A reduction in ejection force was obtained when such samples were compressed in a clean die.

At all values of applied force greater than 700 kg, ejection was facilitated by an increase in moisture content. Above 1700 kg, applied force, the values of ejection force were higher than expected for moisture contents of

less than 0·55%. Conversely, ejection forces obtained for 2·4% and 10% moisture content were lower than anticipated by extrapolation of the linear portion of the graphs.

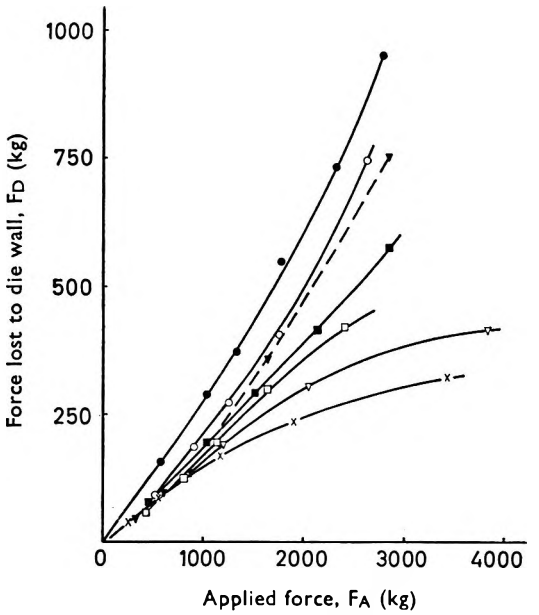

FIG. 2. Effect of moisture on the relationship between applied force and force lost to die wall. Key as in Fig. 1.

For anhydrous sodium chloride, the relationship between the force lost to the die wall and the ejection force at a given applied force was independent of the condition of the die wall (Fig. 4). Moisture exerted a greater effect at the die wall during ejection than during compression, and deviation from the initial relationship for dry material occurred at values of F_E which depended on the moisture content. Above these values of F_E, a common relationship between F_D and F_E was obtained for all samples containing moisture.

CRUSHING STRENGTH

Compacts of anhydrous sodium chloride compressed in a clean die were stronger than those prepared in a conditioned die (Fig. 5). Increases in strength at low pressures were obtained in the presence of 0·02% and 0·55% moisture, compared with anhydrous samples compressed in a conditioned die. Samples containing 10% moisture produced compacts of lower wet strength than 0·55% moisture at all pressures. At high pressures, all samples containing moisture produced compacts of lower strength than the anhydrous material.

The desiccation of moist compacts produced a strength increase which was proportional to the amount of moisture remaining after compression.

During the compression of samples containing 10% moisture, saturated solution of sodium chloride was expelled from the die and at a mean applied pressure (P_M) of 3000 kg/cm^2 only 3% moisture remained in the compact. The percentage increase in strength on drying of compacts compressed in the presence of 10% original moisture was thus greater at low pressures.

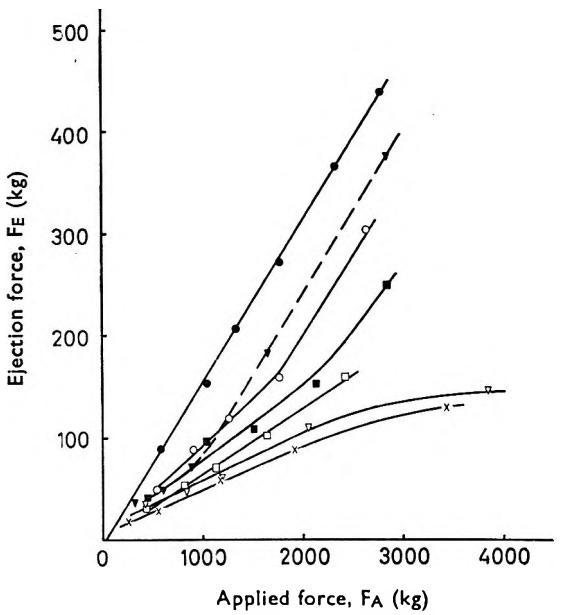

FIG. 3. Effect of moisture on the force required for ejection. Key as in Fig. 1.

Discussion

Conflicting results have been reported concerning the relative importance of the frictional effects at the interparticulate boundary and the particle-die wall interface (Bal'shin, 1938; Seelig & Wulff, 1946; Kamm, Steinberg & Wulff, 1947; Torkar, 1956). Carrington (1958) found an increase in force lost to the die wall when radial movement of particles towards the die wall was facilitated by interparticulate lubrication or punch face lubrication, or both. When lubricant was present at *all* interfacial junctions, a decrease in die wall reaction compared with an unlubricated system indicated that the die wall effect was greater than the interparticulate effects.

The increase in punch force ratio in the presence of 0·55% moisture at low applied force (Fig. 1) may be explained by a reduction in friction due to a lubricant moisture film at the die wall. The force lost to the die wall increases with applied force as the area of compact in contact with the die wall increases. As the porosity of the compact decreases, the void spaces become filled with liquid. Increases in applied force then cause expulsion of liquid to form a continuous film at the die wall. The liquid reduces the

coefficient of friction between particles and die-wall, and also restricts movement of solid into contact with the die. Consequently, for a given increase in applied force there is a smaller increase in contact area, A, between compact and die wall. Assuming that F_D is proportional to A, an increase in applied force then produces only a small increase in F_D.

Lower moisture contents (0·16% and 0·02%) provided less die-wall lubrication at all values of applied forces. No migration of liquid to the die wall occurred even at high applied force, since sufficient void space remained to accommodate the small volume of liquid.

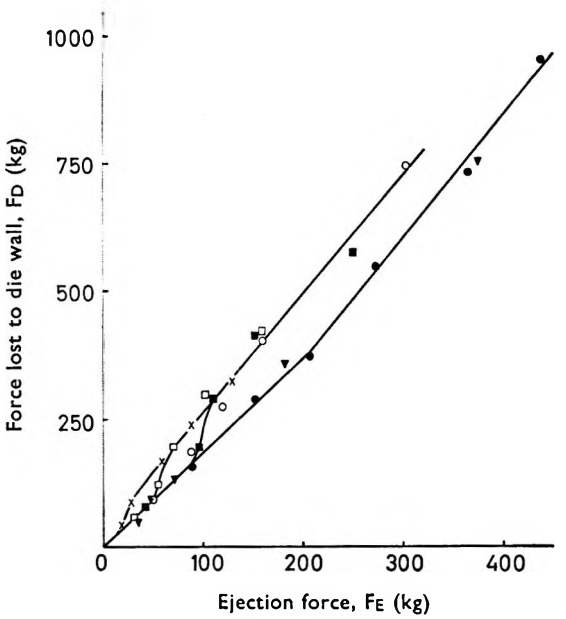

Fig. 4. Effect of moisture on the relationship between the force lost to the die wall and the ejection force. Key as in Fig. 1.

For the theoretical case when all air is preferentially eliminated from the sample containing 10% moisture, it was estimated that when the porosity decreases to 20%, saturated solution will fill the void spaces. In this condition the application of small loads may produce a lateral displacement, or flow, of particles into contact with the die, to yield a higher die wall reaction than for 0·55% moisture (cf. Carrington, 1958). As the applied force increases, liquid is expelled from within the compact and die wall lubrication increases continuously.

The lower values of die wall friction and ejection force for a "clean" die compared with a die which had been "conditioned" confirm that successive compressions increase the extent of die-wall contamination by compressed material. Contamination of the die wall occurs when the shear strength of the bond between the steel die and material becomes greater than that of the material itself. Stephenson (1965) observed a cyclical contamination of the die wall, and a resulting increase in die wall friction, during

compression of anhydrous potassium bromide granules. An increase in moisture content of the granules to 0·5% abolished this effect.

Microscopic examination showed that the presence of water in the sodium chloride caused a rounding off at the edges and corners of the initially cubic crystals, owing to preferential dissolution in these localities. The lubricant effect of saturated sodium chloride solution will be greater between plane or smooth surfaces, whereas points and edges will penetrate the film. The change in crystal shape may thus facilitate movement in the bed and reduction in the incidence of shear failure of crystals.

Increased consolidation of the compact permitted by a lower frictional resistance at the die wall explains the increases in strength of compacts produced by compression in a clean die and in the presence of moisture at low pressures.

A small increase in strength in the presence of moisture may be produced by a surface tension effect which assists the adhesion between particles. This effect will be relatively large where the interparticulate bond is weak and when the number of bonds is small.

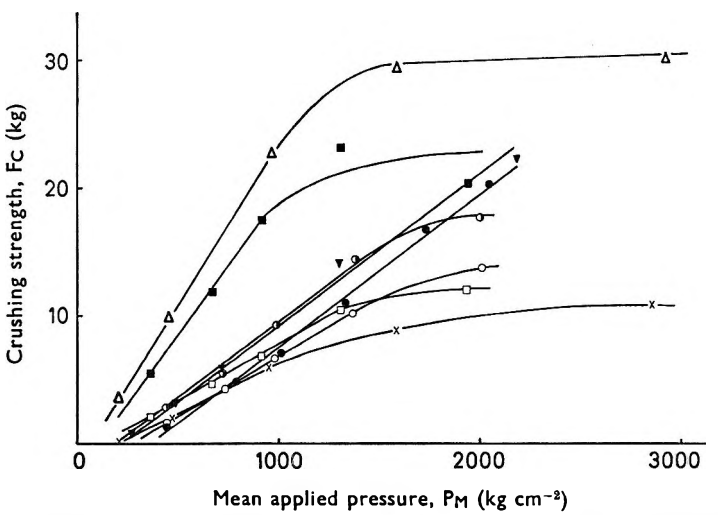

FIG. 5. Effect of moisture on the crushing strength. Moisture %: △, 10 (dried), ×, 10 (wet); ■, 0·55 (dried); □, 0·55 (wet); ◑, 0·02 (dried); ○, 0·02 (wet); ▼, 0 (clean die); ●, 0 (conditioned die).

It appears that 10% moisture exerted a hydrodynamic resistance to consolidation which counteracted the lubricant effects. Despite the low viscosity of the liquid film, lubrication inhibited interparticulate shear forces and thus reduced the amount of bonding which occurred at high pressures. This is in agreement with the results of Strickland, Nelson, Busse & Higuchi (1956) and Lewis (1964) for solid lubricants. Subsequent examination of the fractured surface revealed that failure had almost entirely occurred around the particles (Shotton & Ganderton, 1961) in compacts prepared at low pressure from samples with 10% moisture. At

higher pressures, expression of liquid from the compact permitted interparticulate contact, and evidence of fracture across the crystals increased. Decreasing moisture content resulted in a greater incidence of cross-grain fracture associated with an increase in strength. Anhydrous sodium chloride, compressed at high pressures, showed only cross-grain fracture.

Microscopic examination of the dried compacts confirmed that the increases in strength are mainly due to recrystallisation at the crystal boundaries and in the void spaces. Surface tension forces will produce a tendency for the solution to flow into positions where crystallisation will produce a maximum effect, i.e. at the angles of contact between crystals. Crystallisation of the saturated solution occurring at discontinuities on the crystal surface will effect an increase in crushing strength because it is at such faults that propagation of fracture usually originates (Griffith, 1920).

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Radial voidage variation in randomly-packed beds of spheres of different sizes

K. RIDGWAY AND K. J. TARBUCK

The packing arrangement of the particles in a tablet die, before pressing, has no hitherto been considered as a variable in the tabletting process. Because of the complexity of this subject, the present investigation has been restricted to mono-sized and binary mixtures of spheres in a cylindrical container. The container could be revolved at speeds in excess of 1,000 rpm so that the thickness of an annular layer of water could be measured. From this, the voidage at any radial position could be found. The plot of voidage against distance from the cylindrical wall is a wave form which is damped out after about 5 particle diameters. For packings of mono-sized spheres the oscillations are regular. There appear to be two kinds of binary mixtures, those in which the particle sizes are similar, and those where the diameter ratio is greater than 0·4. The voidages calculated by suitably weighting the values for each component are in agreement with the measured voidages of the first kind but not with those of the second. Because there is an initial distribution of voidage, it is concluded that there must be some radial movement of the particles during the compacting operation.

SINCE the mechanics of random packing of even the simplest forms of particles are not fully understood, any investigation should aim to make measurements on a simple system. Spheres in a simple cylindrical container constitute such a system, and they simulate the situation in a die into which powder or granules have been fed, before compression is applied. For the instance of the packing of mono-sized spheres in a cylindrical container, Scott (1960) has shown that there are two discrete values of the voidage (i.e. the ratio of free space to total space) of the bed, namely 36·3% and 39·9%. These values correspond to "tight", i.e. vibrated or tamped, and "loose", i.e. poured, random packings. The values refer only to the voidage of an infinite array, or alternatively, the central portion of a bed in a large container. They were obtained by extrapolation from the results for a series of beds of known bed diameter: particle diameter ratio. This extrapolation was necessary because at the walls of the container the particle packing is different from that in the bulk. Thus very close to the wall the voidage is high, simply by virtue of the geometry of the sphere and the relative flatness of the container wall (see Fig. 1). The pattern which the voidage follows along a radial line from the wall to the centre of the bed has been determined by Benenati & Brosilow (1962) and Roblee, Baird & Tierney (1958) for a number of values of the (bed/particle) diameter ratio. The present results are in agreement with previous work for mono-sized spheres [see Figs 3 (a), 3 (g), 4 (a) and 4 (g)].

Because the container wall is a smooth surface, the first layer of spheres tend to align themselves in a more or less close packed arrangement. All the spheres in this first layer touch the wall, so that their centres all lie at the same distance from the wall. The first layer is thus well-defined. This arrangement then forms the surface on which the second layer of

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spheres forms and consequently this layer is less ordered than the first. Following this principle, each successive layer is more and more random, until eventually the state of complete random packing is reached.

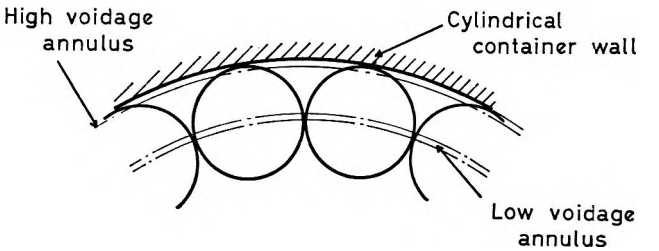

FIG. 1. Two spheres adjacent to a cylindrical wall. The voidage is unity close to the wall and decreases to a minimum at one sphere radius from the wall.

Along any radius of the cylinder there is thus a damped oscillation in the local voidage which can persist for up to five sphere diameters. This fact, it is believed, has not been considered in any tabletting research. The effect is of course dependent upon the size of the powder or granules in the tablet die. For example, Train (1956) used powders of about 200 mesh (aperture 3×10^3 inch) in punch and die sets of 5·68 cm diameter. In this instance the powder may be considered to be bounded by a *flat* die wall. However, many industrial tabletting operations employ 20 mesh granules in dies up to about $\frac{1}{2}$ inch diameter. Here the wall effect may well extend across the entire die charge.

Experimental

The technique used by Roblee & others (1958) and also by Benenati & Brosilow (1960) consisted of filling the interstices of a bed of wooden or lead spheres with a material such as wax or an epoxy resin to form a solid cylinder, which could later be machined on a lathe. The turnings of the annular cuts which were then made were collected and weighed and this enabled the voidage of the annulus to be calculated.

A method adopted by Shaffer (1953) consisted of the incremental filling with water of a packed drum with its axis horizontal. From measurements of liquid level in the drum and the volume of each increment, he was able, by a lengthy calculation, to find what the radial voidage distribution must have been to give the experimental readings which were obtained on *horizontal* layers. However, the calculation had to include the assumption that those parts of the second horizontal increment which were at the outer edges had the same voidage as had been determined for the first horizontal increment. Similarly, the results for the central part of the third increment depended upon taking the values obtained for the first and second. After four increments the results were valueless. We can confirm that this technique has this inherent drawback. It was therefore decided to employ a new technique using centrifugal force. If a packed bed contained in a cylindrical drum were rotated about its

axis at sufficient speed any liquid present would form an annular layer at the surface of the cylinder. The required measurement is then the increase in thickness of this annular layer when a known volume of liquid is added. This gives a measure of the voidage in this thin annular region; this measure is completely independent of the voidage in any other part of the bed and there is thus no cumulative error.

The general arrangement of the apparatus is shown in plan in Fig. 2. It consisted of a 6-inch diameter horizontal cylinder of duralumin, mounted in two 7-inch precision ball races which were an interference fit with its outer surface. The accuracy of the voidage determination depends largely on smoothness of rotation of the drum. On rotation by hand the maximum deviation of the inside surface along the length of the drum was $\pm 1{\cdot}8 \times 10^{-3}$ inch. The overall drum length was about 12 inches.

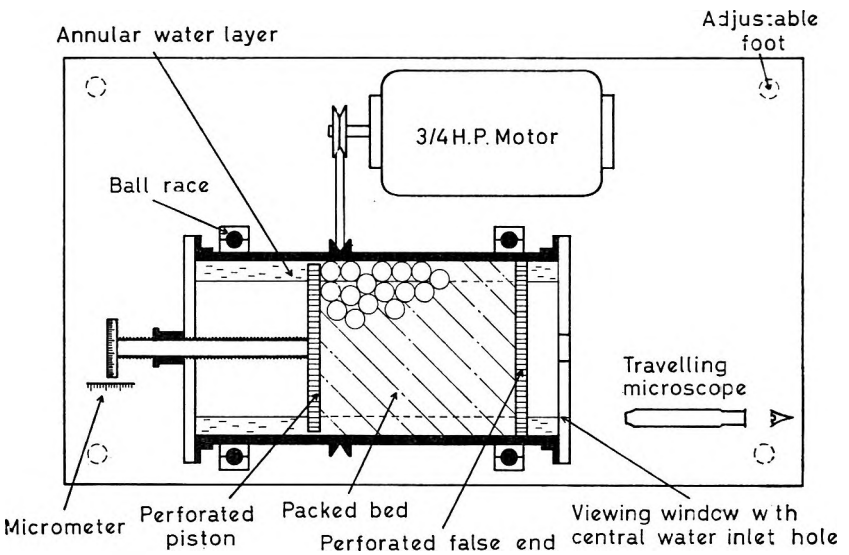

FIG. 2. Plan view of the drum and drive motor. The spheres are compressed into the right hand end of the drum by the piston. Water is added and the water surface in the double window is observed through the microscope.

The required number of spheres are packed into the drum, which can be vibrated by means of an out-of-balance weight rotated by a small subsidiary motor (not shown). The perforated piston is moved in by means of the threaded rod on which it is mounted, until the spheres have been compressed into the minimum volume which they will adopt under these conditions.

The drum initially contains a mixture of ethylene glycol and water of the same density as the spheres. This removes gravitational effects and enables the spheres to be thoroughly mixed before they are compressed by the piston. The glycol–water mixture is then run out and the bed dried by spinning for 10 min. A voidage determination is then made

by adding water, whilst the drum is rotating, through an axial hole in the end of the drum. To enable the thickness of the annular water layer to be clearly observed one end of the drum is double, and consists of a plain outer observation window slightly separated from a perforated inner window which forms one end of the packed bed. Both are made of perspex, and the space between them allows a clear view of the liquid surface.

The drum is rotated by a $\frac{3}{4}$ h.p. DC motor and V-belt drive. Drum speeds between 1,100 and 1,400 rpm are used. At these speeds the deviation from circularity of the meniscus is a few thousandths of an inch, and since the difference of two readings is taken, this small error is balanced out. Because of the bulk of the drum and the relatively high speeds, all plugs, bolts, etc., were balanced by similar plugs and bolts diametrically opposite to prevent vibration. Runs were started at 1,100 rpm, and were usually carried through at this speed; when minor resonances occurred, the drum speed was changed to remove them. In addition, when the meniscus was close to the axis of the drum and the centrifugal force became smaller it was necessary to increase the speed to 1,400 rpm. The drum speed was checked frequently by a tachometer. The position of the water surface was determined by a travelling microscope which traversed horizontally along the diameter of the drum.

Results

The voidage at different distances from the wall of the drum was then determined by following the meniscus movement for 50 ml increments of water. This volume represents an optimum. The incremental volume should be kept small in order to give voidage values which approximate to the values at points. However, the change in thickness of the layer is measured more accurately if the volume added is larger. A compromise has to be made, and a change in thickness of 2 mm was chosen.

In this way, radial voidage variation plots have been obtained for polythene spheres of 8, 9, 10, 12 and 20 mm nominal diameter (plots of the latter four are given in Figs 4 *g*, 3 *g*, 4 *a* and 3 *a* respectively). Plots were also obtained for binary mixtures of 10 and 20 mm balls, and for 9 and 12 mm balls over a wide range of mixtures (Figs 3 *b*, *c*, *d*, *e*, *f* and 4 *b*, *c*, *d*, *e*, *f*). Replicate determinations of most of the curves were made. The replications were of two types (*a*) repeat runs on the undisturbed bed of spheres and (*b*) repeat runs after removing the spheres and repacking the bed. The mean deviation of the experimental points from the best curve which could be drawn through them was not greater than 0·02 units of voidage, up to a distance of 4 cm from the drum wall. Beyond this distance the accuracy decreases.

Discussion

The common feature of all plots, and particularly those for mono-sized spheres, is a regular damped oscillation in voidage. A minimum voidage occurs at a distance corresponding to about half a ball radius from the

wall, i.e. at the centre of the first layer of balls. The first maximum occurs at something less than one ball diameter and the second minimum at the centre of the second layer ball. Because of the increasing degree of randomness, the waveform is gradually damped out.

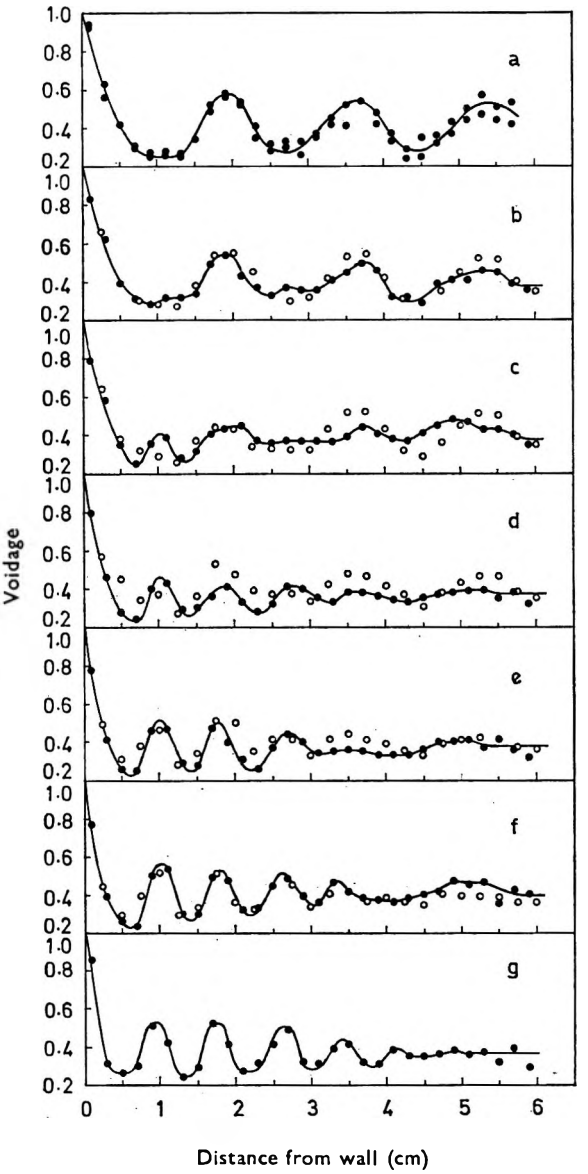

FIG. 3. Radial voidage distribution for mixtures of 10 and 20 mm spheres in a cylindrical container. ●, experimental. ○, calculated by suitably weighting values for the individual components. Mixture composition (% 10 mm by number): a, 0; b, 33·3; c, 58·4; d, 85·7; e, 95·3; f, 99·1; g, 100.

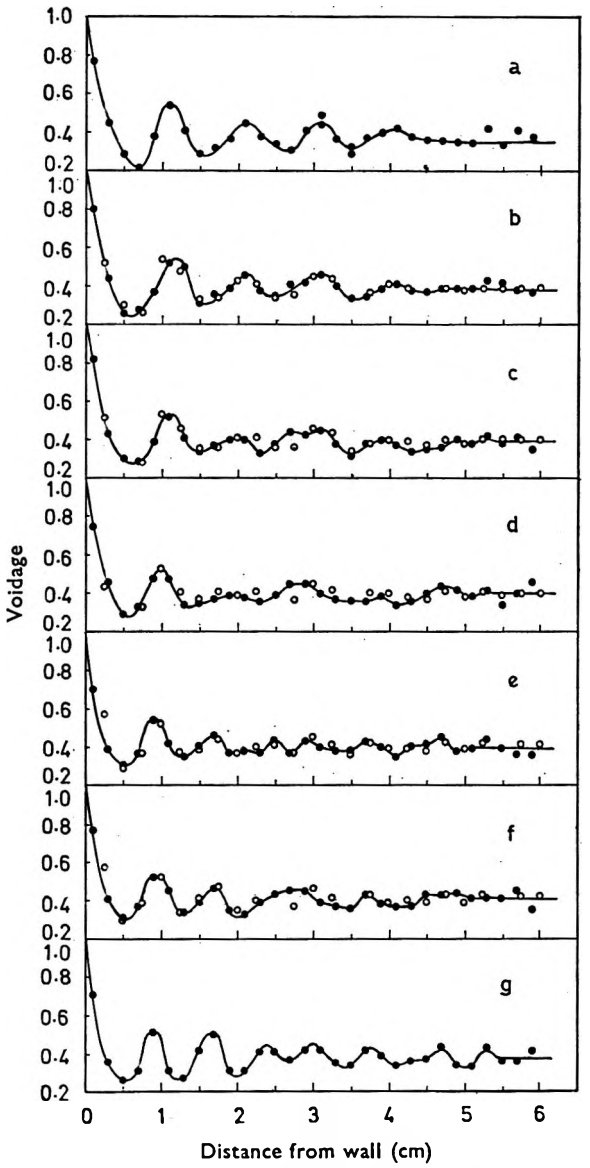

FIG. 4. Radial voidage distribution for mixtures of 9 and 12 mm spheres in a cylindrical container. ●, experimental. ○, calculated by suitably weighting values for the individual component. Mixture composition (% 9 mm by number): a, 0; b, 12·2; c, 31·9; d, 63·7; e, 80·6; f, 89·6; g, 100.

There is an obvious difference between this idealised investigation using spheres in a cylindrical drum and the manner in which non-spherical particles pack in a tablet die. The degree of irregularity of the particle will affect the packing to a great extent. Roblee & others (1958) have

shown that with cylindrical particles (length = diameter) the voidage becomes constant after about three particle diameters only. With Berl saddles, a packing especially designed for distillation columns to give a uniform bed, the voidage becomes constant after about one diameter. No such particles exist naturally, however, and it seems likely that for the irregular particles generally encountered (i.e. when the sizes in three perpendicular directions are similar), the voidage variation dies out after about three to five equivalent diameters.

A voidage plot with the distance measured in terms of particle diameters shows that the curves for different sizes of sphere are similar (Fig. 5). Theoretically, these curves should be calculable, but the problem is one of three-dimensional statistics and as such is still unsolved.

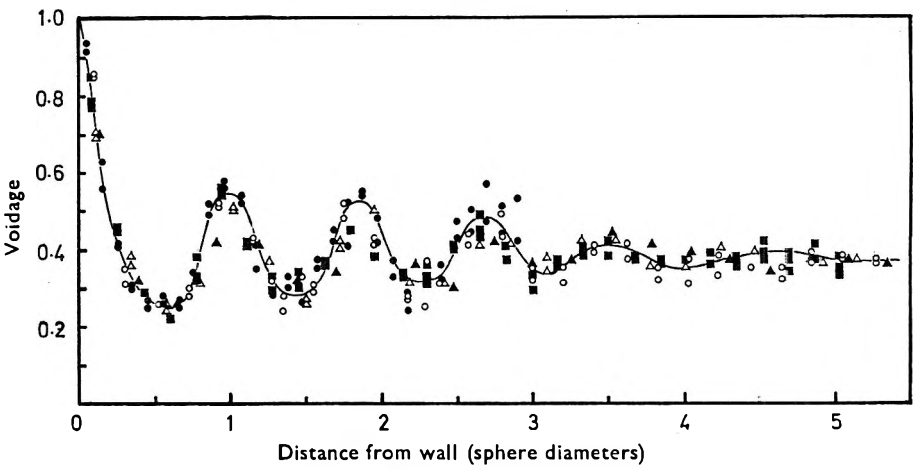

FIG. 5. A plot of radial voidage variation against distance from the drum wall measured in sphere diameters. ▲, 8 mm spheres. △, 9 mm spheres. ○, 10 mm spheres. ■, 12 mm spheres. ●, 20 mm spheres.

The two sets of binary mixtures were chosen to typify instances where the components are of markedly different size (Fig. 3) and where the sizes are similar (Fig. 4). There are two modes of packing for binary mixtures. The smaller spheres can fill the interstices of the larger spheres, and so produce a mixture of low voidage; or, alternatively, the smaller spheres can force apart the large spheres and produce a mixture of higher voidage as in the mixture of 9 and 12 mm balls (Fig. 4). The voidages of these mixtures correspond closely to the voidages calculated from values taken from graphs for the pure components, suitably weighted by the volume fraction.

As would be expected, all the curves for mono-sized spheres are similar, and have five clear minima except for the 20 mm spheres, Fig. 3 *a*, where the ratio of drum diameter to sphere diameter is less than 10 so that five minima cannot be fitted in. The packing thus retains remnants of the layer structure, induced by the wall, up to distances of about five sphere diameters. The mixtures all show a more rapid decay of the

oscillation in voidage and it might appear that mixing spheres of different sizes causes a more rapid approach to randomness. That this is not necessarily so is indicated by the close agreement of the calculated points with the irregular curves in most instances. These points are calculated by summing the product of the voidage due to each pure component at any particular distance from the wall and the volume fraction of that component in the mixture. It thus seems that the different sizes of spheres exert their separate effects even when mixed with other sizes.

A useful practical result may well follow from curve 4 *d*, which is for 42·5% by volume of 9 mm spheres mixed with 12 mm spheres. The voidage fluctuations in this particular mixture become small immediately after the first complete oscillation. This could mean that a more uniform fill of a die would result from a mixture of two closely-sized fractions of granules. Whether this correlates with a better tablet in practice has yet to be tested.

It is generally thought (Kamm, Steinberg & Wulff, and others) that in the compaction of a powder mass in a die, there is no significant radial movement when the die walls are well lubricated. When this is so the powder is compacted in the form of a plug, any straight line parallel to the axis of the plug before compaction remaining undeviated after compaction. This, of course, does not mean that there is uniform compaction along the length of the line; Train (1956) has shown that distinct density patterns exist within a compact for a particular compacting pressure. (In this nomenclature, density = fraction of solids present = 1 − voidage.) It seems apparent that if any radial movement does occur, then it depends on the space available within the bed, i.e. on the radial voidage distribution. Since there is generally more free space near to the wall than in the centre of the bed, it would be expected that some outward radial motion must occur when the bed is compressed to a uniform high-density tablet. Furthermore, the compact should still show signs of annular layers of particles, although obviously the waves of the voidage distribution plot will be largely damped out. This formation can be seen in the lead shot compacts of Hersey (1960).

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The use of photoelastic techniques in the measurement of die-wall stress in tabletting

K. RIDGWAY

A new technique is described for the observation of the stresses acting during a compaction operation. Pharmaceutical materials have been compressed in a Perspex die and viewed by polarised light. By using ancillary Perspex strain gauges both radial pressure and shear stress at the die wall may be determined from the interference pattern. Although Perspex is not a strong material, pressures up to about 12 tons/in² could be contained, and a hard tablet extracted from the die. The entire pressing operation is recorded on ciné film for later analysis.

VARIOUS authors have attempted to measure the variation of radial stress along the cylindrical surface of a compact, and to relate it to the gradual decay of pressure away from the face of the punch. Nelson (1955) has constructed a special punch and die assembly in which a movable section of the die wall is connected to a load cell. With this arrangement he was able to determine die wall pressure, not at a particular point, but only in a region. Windheuser, Misra, Eriksen & Higuchi (1963) made experiments in which a strain gauge was attached to the outside of the die in a zone where the wall thickness had been reduced sufficiently to allow deflection under radial pressure. We have considered the possibility of constructing a ring of piezo-electric material which would be recessed inside the die and would effectively form a part of the die wall. Radial pressure would then be converted into a voltage reading. However, all three methods only record the radial pressure over a comparatively large region of the die wall.

By using photoelastic techniques however, it seemed that readings which approximated more to point values could be obtained. We found that powders could be compressed in small perspex dies between ½ inch diameter duralumin punches in an ordinary bench vice and up to about 15 fringes could be obtained in the Perspex before cracking took place.

In conventional photoelastic measurement, an araldite model of the system to be analysed is made. Araldite is a very sensitive material, in that stresses of a few tens of pounds per square inch produce several interference fringes. This would have been useless for the present purpose, because although a model die could have been made, no model powder could. Powder behaviour is a non-linear function of the applied stress: the behaviour at 10 lb/in² stress is qualitatively unlike that at 10,000 lb/in².

Experimental

It has proved possible to compress a tablet in a perspex die, to remove it by means of a duralumin ejector, and to examine its properties. Ejection is difficult, though not impossible, because the strain in a perspex die is much larger than that in a steel die. Usually some degree of lamination or capping of the tablet takes place, indicative of a high degree of compression. Compaction of powders was carried out using a hydraulic

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ram up to a pressure of 60 lb/in^2 (equivalent to 12·7 tons/in^2 on the compact).

The general arrangement of the apparatus is shown in Fig. 1. The Perspex die is mounted between the solid pillar-like extensions fixed to the upper and lower platens of a Tangye hydraulic press, capable of exerting a force of 100 tons. The lower pillar sits on a steel bridge, through which the optical bench passes. The gauge recording pressure in the hydraulic system is fitted with a flexible connecting tube so that it can be positioned on the right of the die. To the left of the die, a micrometer dial gauge, mounted on a magnetic holder, is used to record the lower punch movement during the tabletting operation. The optical bench, 6 feet long, carries a tungsten filament photoflood lamp operated from the mains through a variable transformer and underrun at about 140 V. Light from this passes through a 9 inch diameter condenser lens, through the polariser and the Perspex die, and then on through the analyser to a ciné camera. Stray light is eliminated by a black cloth draped over the press, and by a large internally-blackened cardboard tube covering the distance between the press and the camera. To make the dial and pressure gauge readings visible, it was necessary to provide front lighting by means of small spot-lights.

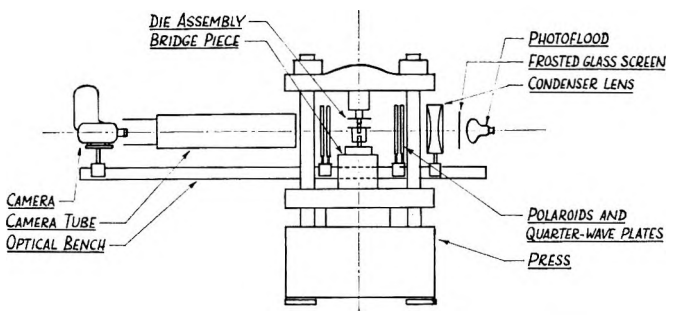

FIG. 1. General arrangement of the apparatus. Compression is applied to the lower punch of the punch and die assembly by the lower platen of the hydraulic press. The optical bench carries the light source, polaroids and camera and keeps them in accurate alignment. The bridge piece carries the force from the lower platen to the punch, whilst preventing the movement being transmitted to the optical bench.

The arrangement of the die is shown in Fig. 2. The die is made from a cylinder of Perspex 2 in long and $2\frac{1}{4}$ in in diameter, with two flat surfaces on it to give a clear view of the central $\frac{1}{2}$ in diameter hole. A graticule of lines is ruled on one of the flat surfaces to provide reference zeros for fringe measurements. The upper and lower punches are made of free cutting stainless steel, and are a sliding fit in the central hole of the die. A duralumin disc fits around the top punch and rests on top of the Perspex die block. Spacers are sometimes required. Between the disc and a similar one attached to the upper pillar, two small pieces of Perspex are placed; these are so shaped that a vertical force applied to them causes bending in the shank. The shank is sufficiently long to give the characteristic striped pattern of pure bending fringes over a sufficient

length to enable the force to be determined by fringe counting. Compressive force is applied only to the lower punch. Radial stresses in the die give a fringe pattern directly. Any tendency of the die to move upwards due to the imposition of shear forces on the die wall by the moving powder is transmitted through the duralumin disc and appears as a fringe pattern in the small Perspex pieces. Two small triangular prisms are placed one above and one below the die, so that an additional view of the fringe pattern inside the die is obtained on a vertical line.

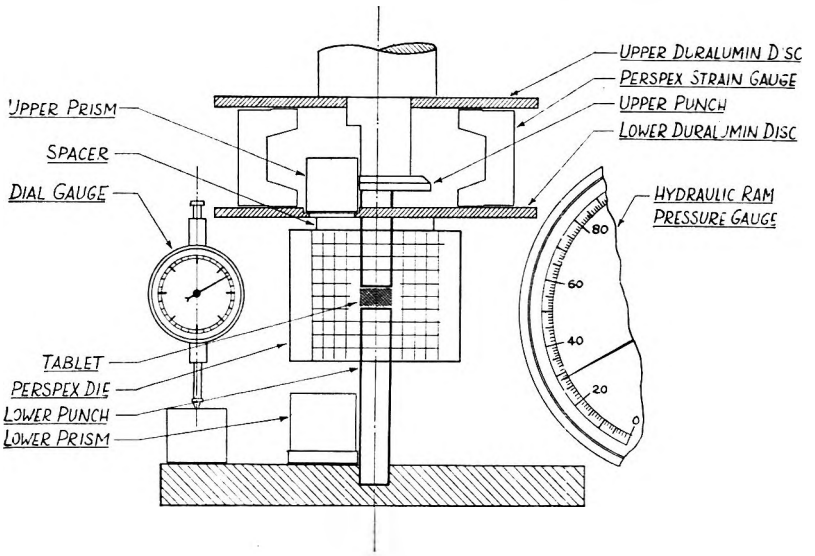

FIG. 2. The punch and die assembly. Radial stress produces fringes in the body of the die. These are viewed directly, and are also inspected along a vertical line by means of the upper and lower prisms which form a periscope. Shear stress at the wall causes upward movement of the die. This is transmitted to the upper Perspex strain gauges, which bend and give fringes in their vertical shanks. Lower punch movement is measured by the small dial gauge, and the force applied to the lower punch is given by the pressure gauge.

A known weight of the powder under study is put into the die, which is placed in position in the press. The lighting and focusing of the camera are adjusted, and the screening for the exclusion of stray light is placed in position. The camera is started, and the press pressure is increased by manual operation of a spring-loaded control lever which operates the valve leading hydraulic fluid to the ram. The pressure is increased at a slow uniform rate; by means of a mirror it is possible to see the pressure and dial gauge faces during operation of the press. An average pressing run takes only a few minutes, and usually occupies about 75 ft of 16 mm Kodachrome 2 ciné film. In the results reported, at the normal speed of 16 frames/sec, the exposure time was 1/40 sec at f.11.

PHOTOELASTICITY THEORY

The basis of photoelasticity is that when a beam of plane-polarised light passes through a piece of transparent plastic material under stress,

the beam is split into two components. One of these components is retarded relative to the other, due to its travelling at a different velocity. At the analyser, the two beams recombine, to produce interference fringes which can be photographed. The relative retardation is governed by the properies of the plastic material (usually expressed as a "fringe constant" C), its thickness, d, measured along the light path, and the difference between the two principal stresses acting perpendicularly to the light path.

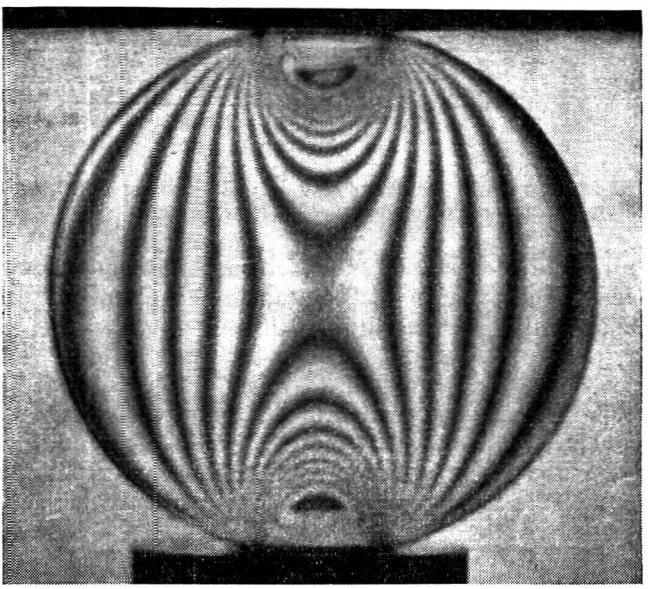

FIG. 3. The fringe pattern obtained when a Perspex disc is loaded along a diameter. For an applied load P, the stress difference at the centre of the disc is $8P/\pi dt$. This enables the fringe constant of the material of the disc to be determined.

Thus

$$\text{relative retardation} = C \times d \times \text{(principal stress difference)}.$$

It can be shown (Jessop & Harris, 1949) that the relative retardation will be such as to cause extinction of a beam of light of a particular wavelength whenever the relative retardation is an integral number of wavelengths: this gives rise, in white light, to coloured fringes called "isochromatics". There will also be extinction wherever the directions of the principal stresses are parallel to the polariser and analyser respectively. Such dark lines are called "isoclinics".

MEASUREMENT OF THE FRINGE CONSTANT

If a disc of the photoelastic material is compressed along a diameter, a fringe pattern is formed as in Fig. 3. The principal stress difference at the centre is known to be $8P/\pi dt$ where P is the applied load, d is the diameter and t the thickness of the disc. Fig. 4 is a plot of fringe order at the centre of the disc against the applied force. The plot is linear over most of its length, indicating that the fringe order is proportional

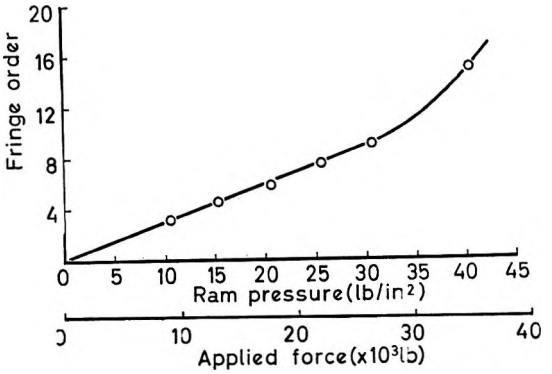

FIG. 4. The fringe order at the centre of a 1¼ inch diameter ¼ inch thick Perspex disc plotted against the applied diametral load.

to the stress until the material is no longer elastic and ceases to obey Hooke's law. The value of the constant is 476 lb/in² per fringe per inch thickness within the elastic limit.

Results

The results presented here are intended only to show the usefulness of the technique.

Fig. 5 shows the general appearance of the fringe pattern in the perspex die when a tablet is under compression. This particular tablet was made

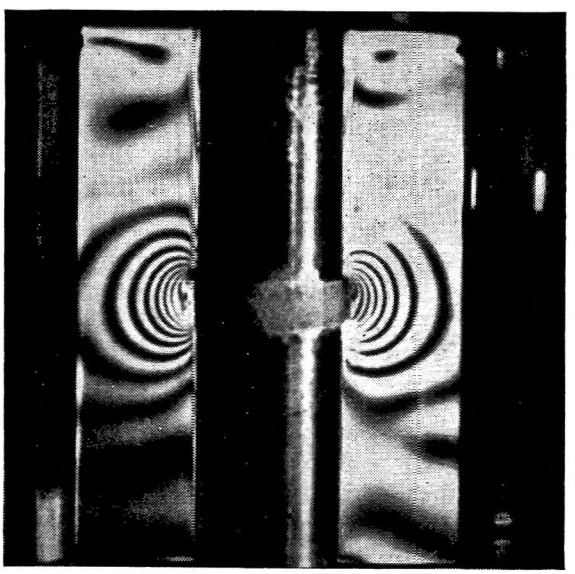

FIG. 5. The appearance of the fringes obtained in the Perspex die when a tablet is being compressed (20–30 mesh aspirin; ½ inch diameter tablet with 7800 lb applied force, equivalent to 39,600 lb/in²)

of 20–30 mesh aspirin, and weighed 1 g. A series of pictures taken as the tablet was compressed enabled readings to be abstracted of the ram pressure and the movement of the lower punch (and thus the extent to which the tablet was reducing in thickness as pressure was applied). The fringe order at this tablet edge was counted and enabled the plot in Fig. 6 to be made.

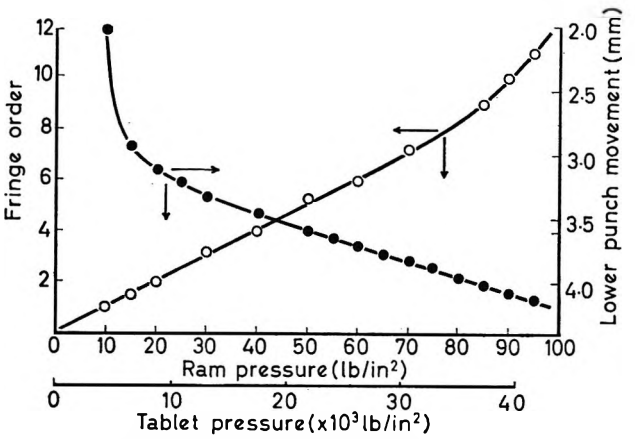

FIG. 6. Fringe order in the die wall immediately adjacent to a tablet (a measure of the radial pressure) plotted against pressure applied. The tablet is that shown in Fig. 5.

The fringe order increased as the punch pressure, and therefore the radial pressure exerted on the die-wall, increased. To assess the constant of proportionality a known radial pressure was applied to the die-wall by applying the punch pressure to a liquid confined in the tablet space. Since the pressure distribution must then be hydrostatic, the radial die-wall pressure must be equal to the applied punch pressure. The difficulty of confining a liquid in the die at the high pressure required was surmounted by wrapping a small amount of silicone putty in a small sack of chamois leather, wired at the neck. With an upper punch recessed in the centre to accept the wired neck of the bag, this was compressed up to a ram pressure of 30 lb/in². The fringe pattern was similar to that obtained on compression of a powder, but the fringe order was approximately three times as great for the same applied pressure, over the restricted range which could be covered before leakage occurred. Thus the radial pressure exerted by the tablet was about one-third of the applied pressure. This agrees with the finding of Nelson (1955) that in the compression of unlubricated sulphadiazole the radial die-wall pressure is about 30% of the applied punch pressure.

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Some angular properties of magnesia and their relevance to material handling

T. M. JONES AND N. PILPEL

By measuring the angular characteristics of magnesia over a range of particle sizes from 30–3000 μ, it has been possible to determine the factors which influence the shapes of heaps, cones and wedges of the material, formed under a variety of experimental conditions. The relevance of the measurements to quality control, flowability, hopper and chute design and material handling is discussed.

WITH the increasing use of particulate solids in pharmaceuticals, interest has been stimulated in the angular properties of materials in granular and powdered form.

Several authors have outlined the methods available for measuring these properties (Zenz, 1957; Train, 1958; Brown, 1961; Pilpel, 1964) but the choice of a method for use in control investigations is often arbitrary. Consequently, the results obtained bear little relationship to the handling characteristics of the material concerned. Furthermore, little is known of the interrelation between the results obtained by the various methods.

In the present work an attempt has been made to compare the results of a number of these tests for one material over a range of sizes, the physical properties and flow characteristics of which are now established (Jones & Pilpel, 1966a, b). From these results it has been possible to evaluate the techniques critically and to note their application to handling characteristics.

Experimental

Determinations were carried out on close cut sieve fractions of free flowing magnesia, in the range 30 μ to 3000 μ. The physical properties of these fractions have been previously reported (Jones & Pilpel, 1966a).

ANGLES OF REPOSE

Natural angles of slip, θ_1, were measured by a fixed cone method using 50 g of powder and funnels with an angle of 45°; orifice diameters (1) 0·4 cm, (2) 0·7 cm; stem lengths 5·6 cm, terminating 3 cm above a horizontal surface (Train, 1958; Nash, Leiter, Johnson, Stender & Zeller, 1963; Kawai & Hasegawa, 1964). Static angles of repose, θ_2, were measured by the fixed base and cylinder method (Pilpel, 1964) using bases of diameter 3·69 and 9·00 cm with loads of 35 g and 400 g of powder respectively. Consolidated static repose angles, θ_3, were obtained by measuring a two dimensional drained heap in a rectangular box with a glass front (Jones & Pilpel, 1966b), the wedge length being 4·8 cm.

The angles were calculated from a knowledge of the heights, h, and the radii, r, of the heaps by the formula

$$\theta = \tan^{-1} \text{ h/r}$$

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where h and r were measured with a cathetometer. Between 6 and 12 replicates were determined for each sample, the results being expressed as mean values or as a range when the values were very varied.

In some instances it was convenient to measure the length of the sides of the cones, s, using dividers, readings being taken at four positions separated by 90° of arc. The angles in this case were calculated from

$$\theta = \cos^{-1} r/s$$

It was also possible to calculate the natural angle of slip from a knowledge of the mass, M, the bulk density, ρ_B of the sample, and the diameter of the cone base, d. The following formula was used:

$$\theta = \tan^{-1}\frac{24M}{\pi\rho_B d^3}$$

[This is derived from the fact that the volume of a cone $=\frac{1}{3}$ the volume of a cylinder of the same height standing on the same base]. With this formula, difficulties in measuring the height of cones because of flattened apices or small values of h, can be eliminated.

ANGLES OF FRICTION

These were determined by measuring the angle from the horizontal at which shearing or slipping occurred in heaps or beds of material. Three methods were used:

(1) Tilting a heap, formed by the fixed cone method as above, and noting the angle of inclination, α_1, at which shearing occurred (Lowes & Perry, 1965). This yielded a dynamic internal shear angle.

(2) Preparing beds of material by dredging from a height of 5 cm onto a glass plate (cleaned with chromic acid), and weighing the quantity of material falling off at each angle of elevation (Krishna & Rao, 1963). The angle of sliding friction, α_2, was taken as the point of maximum inflexion in plots of weight versus angle. Measurements were made on beds several particles thick, α_{2_1}, and on beds one particle thick, α_{2_2}. The mean angle for single particles, α_{2_3}, was also noted.

(3) Rotating the material in a drum of diameter 13·6 cm, width 2·7 cm, at a speed of 2 or 4 rpm (Franklin & Johanson, 1955). Four angles were measured—the static angle for loosely packed, α_{3_1}, and compacted, α_{3_2}, samples, the surface kinetic angle, α_{3_3}, and the internal kinetic angle, α_{3_4}.

The results of the measurements on the different size fractions are summarised in Figs 1, 3 and 5.

Smooth curves have been drawn through experimental points although it does not necessarily follow that the angular characteristics are a unique function of particle size.

Discussion

The forces involved in the formation of heaps, cones and wedges of particles are mechanical, gravitational and interparticulate (frictional and cohesive) and attempts have been made to correlate the angles of the heaps with these fundamental forces (Dawes, 1952; Lowes & Perry, 1965).

Fig. 1 shows that the natural angle of slip, θ_1, remains appreciably constant over the range of particle sizes investigated. This method is essentially a measure of the packing characteristics and hence the shape and rugosity of the material. Since θ_1 is formed under kinetic conditions, it may be assumed that in this measurement the effect of interparticulate forces is largely overcome.

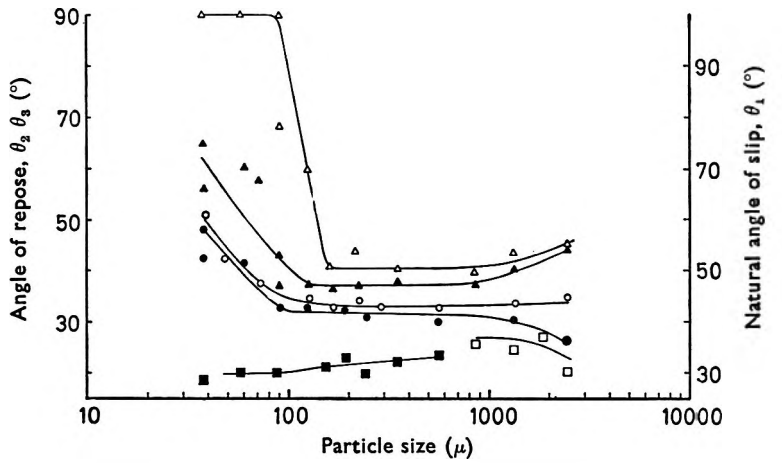

FIG. 1. The change in angle of repose with particle size. ■, θ_1 using funnel (1); □, θ_1 using funnel (2); ●, θ_2 (large base); ○, θ_2 (small base); ▲, θ_3 (loosely packed); △, θ_3 (consolidated).

The cones formed in the static methods are produced by the shearing forces in the mass surrounding them. At small particle sizes, the interparticulate forces oppose these shearing forces and consequently more material is held in the heap than would otherwise be the case. This may explain why the angles θ_2 and θ_3 show a distinct rise in value as the particle size decreases below 100 μ (Fig. 1).

The consolidated angles, θ_3, are even higher than the static angles of

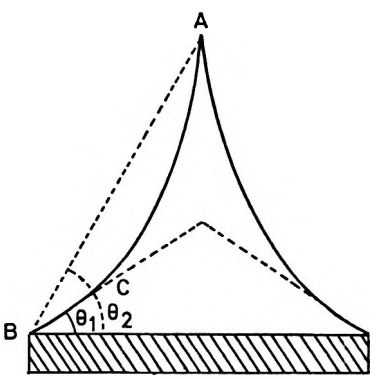

FIG. 2. Diagrammatic representation of false angle of repose.

repose, θ_2, since in the closer state of packing the forces between the particles are enhanced and can act more fully.

Heaps formed by material possessing strong interparticulate forces are often irregular (Craik, 1958) and seem to consist of a cone of angle θ_1 surmounted by a peak (Fig. 2). Similar observations have been reported by Dawes (1952). It is possible that these false peaks are produced by the weight of material enclosed by ABC (Fig. 2) exerting a shearing force greater than the force required to hold the material in position. The magnitude of such a shearing force is reflected in the values of α_1 (Fig. 3), the angle (about 10°) being small by comparison with the angle ABC (between 15° and 30° for $\theta_2 \simeq 45°$ and 60° respectively). The limiting angles at which monodispersed particles roll down slopes of randomly packed spheres of the same size are between 19·3° and 45° (Train, 1958). The upper limit (45°) is equivalent to the value of $\theta_1 + \alpha_1$, i.e. the angle above which false peaks are formed.

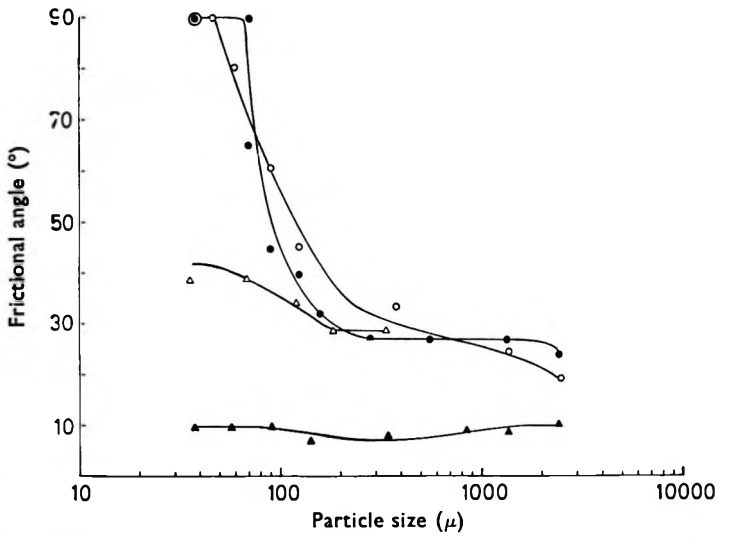

FIG. 3. Variation in frictional angles with particle size. ▲, α_1; △, α_{2_1}; ○, α_{2_2}; ●, α_{2_1}.

Since the false peak may be attributed to the influence of interparticulate and shearing forces, it may be possible to estimate their magnitude from a knowledge of the mass of material in the peak together with the influence of mechanical and gravitational forces on the sample. However, the height of the peak may be affected by disruptive shearing forces during formation and this would be a complicating factor.

Angles of friction, α_{2_1} and α_{2_2}, for beds of solids under dynamic conditions, also increase at small particle sizes (Fig. 3). The angle of friction α_{2_1} is a combination of the angle at which material slides over itself and over the supporting surface, whereas α_{2_2} is the apparent angle at which it slides on the glass only: it can be seen that $\alpha_{2_2} > \alpha_{2_1}$. In characterising

material, it is often necessary to know both α_{2_1} and α_{2_2} since the former can be related to the internal frictional characteristics of beds and the latter to the adhesion of material to a given surface.

Beds of fine material, several particles thick, shear at α_{2_1} to produce beds one particle thick; thus α_{2_1} and α_{2_2} can both be determined in the same test (Fig. 3), two points of inflexion in the weight versus angle plots being observed.

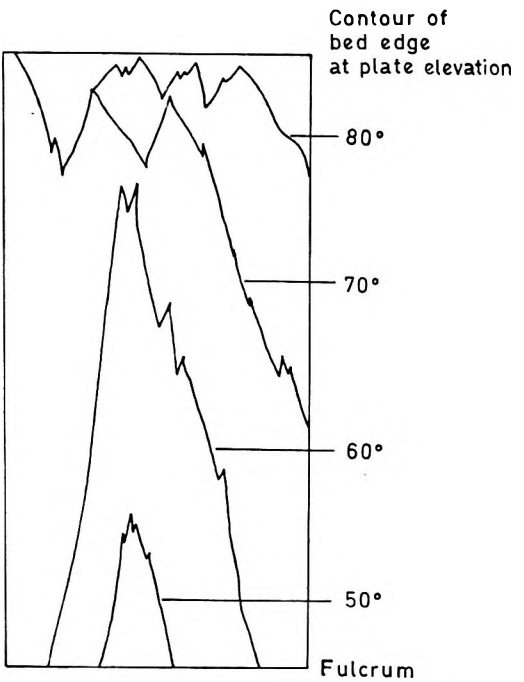

FIG. 4. Triangular shear pattern on tilting bed of single particles. 71μ diameter.

The values of α_{2_2} correspond closely to the mean angles of friction of individual particles α_{2_3} from a given sieve cut. In any bed of single particles there will be a distribution in the values of α_{2_3} due to slight differences in rugosity, shape and size. Thus on tilting, the particles having the lowest value will slide first. If the weight of these particles is sufficient, their downward motion will disturb the particles immediately below them and cause these to slide also. This kinetic disturbance spreads outwards and downwards producing a triangular shear pattern (Fig. 4). As tilting continues, other particles situated higher in the bed also cause propagating collisions with consequent triangular patterns. Similar conditions would arise in beds of mixed particle size or where aggregates have formed in monodispersed beds.

The static angles α_{3_1}, α_{3_2} vary similarly to θ_2, θ_3 (Fig. 5). α_{3_1}, α_{3_2} are the maximum angles which the surface subtends to the horizontal

before movement occurs and this fits the broad definition of angle of repose as the angle of friction taken up by a granular solid about to slide upon itself (Fowler & Wyatt, 1960). The relationship between α_{3_1}, α_{33} and α_{3_4} fits the proposed correlation of Franklin & Johanson (1955); it is thus possible to estimate the internal kinetic angle from a knowledge of the static or surface kinetic angle for sieve fractions where the internal angle is difficult to measure.

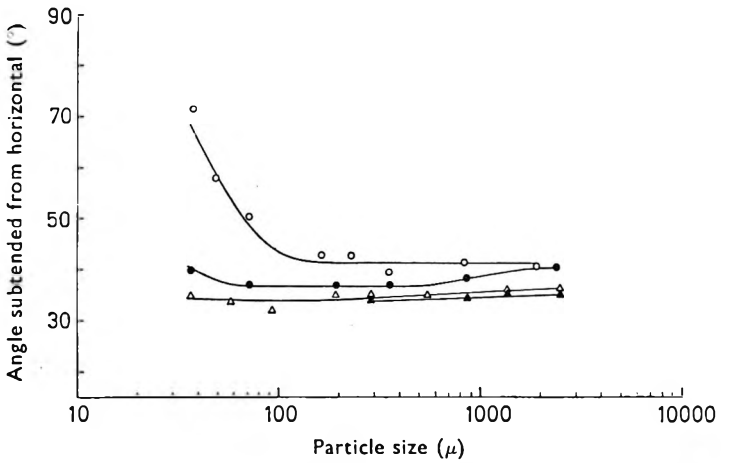

FIG. 5. Variation in angle of friction (α_3) with particle size, ●, α_{3_1}; ○, α_{3_3}; △, α_{3_3} ▲, α_{3_4}.

It can also be seen from Fig. 5 that the value of α_{3_3} does not vary appreciably with particle size. Thus under kinetic conditions the influence of interparticulate forces is outweighed by the kinetic energy of the particles. As the value of the surface kinetic angle is essentially similar to the natural angle of slip, θ_1, the original postulate that in measuring θ_1 interparticulate forces are overcome, appears to be justified.

Application of the above principles to material handling

CONTROL

Angles of repose can be successfully applied to control excessive moisture in powders and granulations (Wolf & Hohenleiten 1945; Craik & Miller, 1958; Fowler & Wyatt, 1960).

Since the presence of fine particles in granulations produces changes in the value of θ (Nelson, 1955; Craik, 1958; Pilpel, 1964) methods which do not cause segregation of material may be used as a control over excessive fines produced during handling.

The extent of segregation can be observed by forming a two-dimensional heap, from a wedge shaped hopper with a slotted outlet. Coarse material with a low angle of friction, α_{2_3}, rolls and slides towards the base (Fig. 6), whilst fine material possessing a high angle of friction remains in the central core. As the heap is built up, distinct striations are observed.

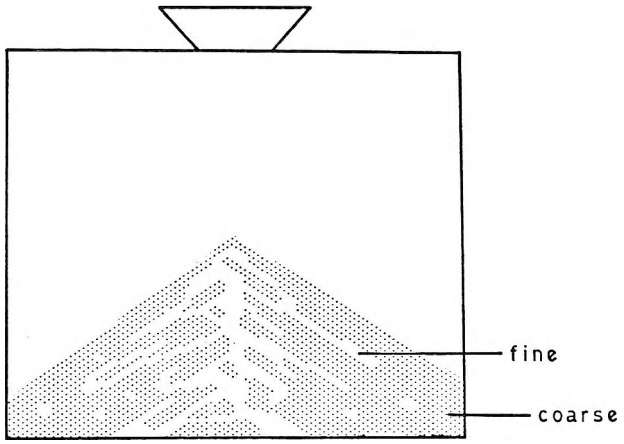

FIG. 6. Two dimensional heap of coarse and fine particles (schematic).

FLOWABILITY

An increase in the static angle of repose, θ_2 or θ_3, reflects a decrease in flow rate (Jones & Pilpel, 1966b).

In general if $\theta < 40°$ a material will flow easily through orifices and from hoppers. When the angle exceeds 50° flow takes place with difficulty and aggregation, "rat holing" or bridging may occur.

Because heaps with $\theta_2 > 40°$ often possess false peaks which may be disturbed during formation, it seems unwise to rely to any extent upon quantitative values in these instances, although qualitatively, dramatic variations in the angle of repose can be identified easily. The values of α_{3_1} however, are more reproducible since they apply to free surfaces. Consequently the values can be used directly and for comparisons between powders.

Clearly if the material is compacted by vibration or ramming, the angles θ_3 and α_{3_2} become relevant (Figs 1 and 5). These are higher than θ_2 or α_{3_1}, particularly at the small sizes. The problems of handling fine particles and of making them flow are often very difficult to overcome as a result of compaction.

Several authors have incorporated values for angle of repose or angle of friction in equations for calculating flow rate (Takahasi, 1935; Shirai, 1952; Zenz, 1962; Kawai & Hasegawa, 1964). From the present work and that of others (Train, 1958; Brown, 1961) it is obvious that these characteristics of a powder are not easily and uniquely definable. Although it may be advisable to include qualified values in flow data as a guide to material characteristics, it appears unwise to use them directly in quantitative flow equations.

HOPPER AND CHUTE DESIGN

The drained angle of repose, θ_3, implicitly defines the dead space in a horizontal based hopper after discharge. However, the static region

during flow has a shear angle much greater than θ_3 (Brown & Richards, 1965) and designing hoppers with base angles $< \theta_3$ does not necessarily give the best flow improvement.

The angles of friction α_1, α_2 and α_3 are useful in deciding the slope of a chute for conveying material. Caution should be observed in interpreting laboratory tests using constructional materials which differ from those used in the plant. Fowler & Chodziesner (1959) have developed an expression to perform such conversions.

FINE PARTICLE HANDLING

The methods used in these investigations are only of real value for relatively free flowing material since only these form good cones and heaps.

For fine particles $< 50\,\mu$, static and dynamic methods produce heaps which do not possess well defined angular properties; angles of friction reach a limiting value of 90° and the free surfaces of rotating beds of fine particles are uneven. In these cases, the split plate method (Dawes, 1952; Shotton & Harb, 1966) and the Jenike Shear Cell (Ashton, Cheng. Farley & Valentin, 1965; Williams & Birks, 1965) have been successfully applied to studies on a variety of different materials.

In conclusion it remains to point out that the relationships between the various angles quoted in this paper are applicable only to the magnesia used. Further work on other materials is required to establish the generality of these relationships.

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Cortisone acetate crystal forms

J. E. CARLESS, M. A. MOUSTAFA* AND H. D. C. RAPSON

Infrared spectroscopy and differential scanning calorimetry data are given for the characterisation of three anhydrous and two hydrated crystal modifications of cortisone acetate. All forms revert to the same form on heating to 200°. The amount of hydration estimated from thermal analysis varies from half to one molecule of water per molecule of cortisone acetate. Methods for the preparation of the five forms are described emphasising the importance of solvent composition, particularly its water content. Interconversion of these forms under various conditions such as grinding, contact with water and heat treatment is discussed.

CORTISONE acetate is reported to exist in different polymorphic forms (Garratt & Marshall, 1954; Callow & Kennard, 1961; Mesley & Johnson, 1965). Published spectra from different sources show distinct differences (Neudert & Röpke, 1957; Tarpley, Yudis, Manowitz, Horrigan & Weiss, 1954; Meda, 1958; Hayden, Sammul, Selzer & Carol, 1962). The characterisation and preparation of these forms has not been adequately described in the literature. There has been much confusion about the number of these forms and the exact methods to be used for their identification. Using infrared spectroscopy and differential scanning calorimetry, the present inquiry has investigated the physical factors responsible for the formation and stability of these different forms. The identification of different crystalline modifications is essential for the study of polymorphic changes of the material in suspension or in the dry state. Methods for preparing the different crystal forms are described in the present paper.

Experimental and results

MATERIAL AND APPARATUS

Two batches of cortisone acetate (British Drug Houses Ltd.) were used and found to belong to different crystalline forms. A third sample was a micronised product from Roussel Laboratories.

Infrared spectroscopy. Spectra were determined with a Unicam SP200 double-beam spectrometer with a sodium chloride prism. A Unicam SP100 grating spectrometer was used to confirm some of the results and to identify effluents collected in a gas cell.

Differential scanning calorimetry. A Perkin-Elmer DSC-1 apparatus fitted with effluent analyser was used (see Wendlandt, 1964). Dry nitrogen at 30 ml/min was used as the carrier gas. The rate of heating generally adopted was 32°/min and sample weight varied from 1–10 mg. The heat of fusion of tin was used to calibrate the response of the calorimeter. The thermal conductivity analyser was calibrated for water vapour measurement by heating varying amounts (0·5–10 mg) of $CuSO_4.5H_2O$

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in the sample pan of the calorimeter. Peak areas recorded were determined by planimeter. For the identification of volatile products evolved from cortisone acetate during heating in the calorimeter, the effluent was passed through a specially designed trap, cooled in liquid nitrogen and the volatile material subsequently transferred to a gas cell for identification by infrared spectroscopy.

Thin-layer chromatography. The technique described by Hall (1964) was used to check the purity of starting materials and to follow the stages in purification by crystallisation or in heating at various temperatures.

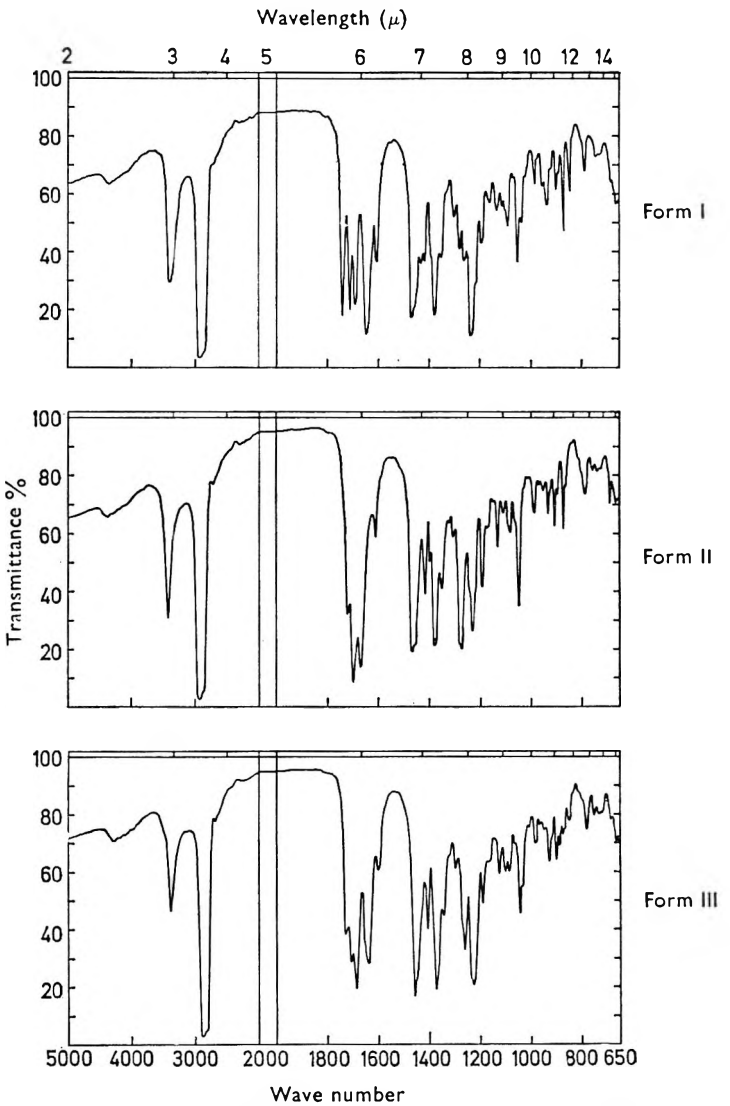

FIG. 1. Infrared spectra of cortisone acetate crystal forms in Nujol mulls.

Nomenclature. The numbering system used for nomenclature is the same as used by Callow & Kennard (1961), except that Forms I and III have inverted notation.

PREPARATION OF THE DIFFERENT FORMS

Form I was only obtained by heating any other form to a temperature of 200°. The solvents used for crystallisation of Forms II–V are as follows: Form II—chloroform, benzene; Form III—30% v/v water in acetone, water at 100°; Form IV—95% aqueous ethanol; Form V—carbon tetrachloride–anhydrous methanol (3:1 v/v). The cortisone acetate was dissolved in boiling solvent and then rapidly cooled in a refrigerator for about 1 hr and the separated crystals stored over silica gel and concentrated sulphuric acid in a desiccator. Prolonged contact of crystals with certain solvents, e.g. carbon tetrachloride or dimethylformamide, led to discolouration and to an increased number of steroid impurities.

CHARACTERISATION OF THE DIFFERENT FORMS BY INFRARED AND DIFFERENTIAL SCANNING CALORIMETRY

Infrared spectra of Forms I–V are presented in Fig. 1. Main characteristics other than those in the carbonyl and hydroxyl stretching regions include a prominent moderately strong single band at 870 cm^{-1} in Form I, a prominent strong band at 1,275 cm^{-1} of higher absorbance than the band at 1,230 cm^{-1} in Form II, and a band at 3,540 cm^{-1} probably due to an enol in Form V.

TABLE 1. QUANTITATIVE DATA FROM THERMAL ANALYSIS

Form		Energy uptake during melting kcal $mole^{-1}$	Energy uptake during loss of solvent kcal $mole^{-1}$	Quantity of water detected mol/mol
I	(raw data)	8·78, 8·56, 9·74	—	—
	(average)	9·03		
II	(raw data)	10·12, 8·83, 9·20	—	—
	(average)	9·38		
III	(raw data)	8·27, 10·21, 9·85	—	—
	(average)	9·44		
IV	(raw data)	8·52, 8·01, 10·12	10·12, 9·81, 9·69	0·58, 0·59, 0·40
	(average)	8·88	9·87	0·53
V	(raw data)	10·42, 7·93, 10·13	8·86, 7·77, 7·82	0·98, 0·98, 0·88
	(average)	9·49	8·15	0·95

Thermal analysis (Table 1, Figs 2, 3 and 4) shows that about 8–10 kcal $mole^{-1}$ are absorbed by all forms on melting, although the exact temperature of peak melting varies slightly from one form to another. In addition, Forms IV and V show a second (endothermic) transition of 8–10 kcal $mole^{-1}$ far below their melting point. This arises from loss of water together with a small amount of carbon dioxide. For freshly prepared

samples of Form IV, it corresponds to half a molecule of water, per molecule of cortisone acetate and for Form V to one molecule of water. Forms I, II and III are anhydrous.

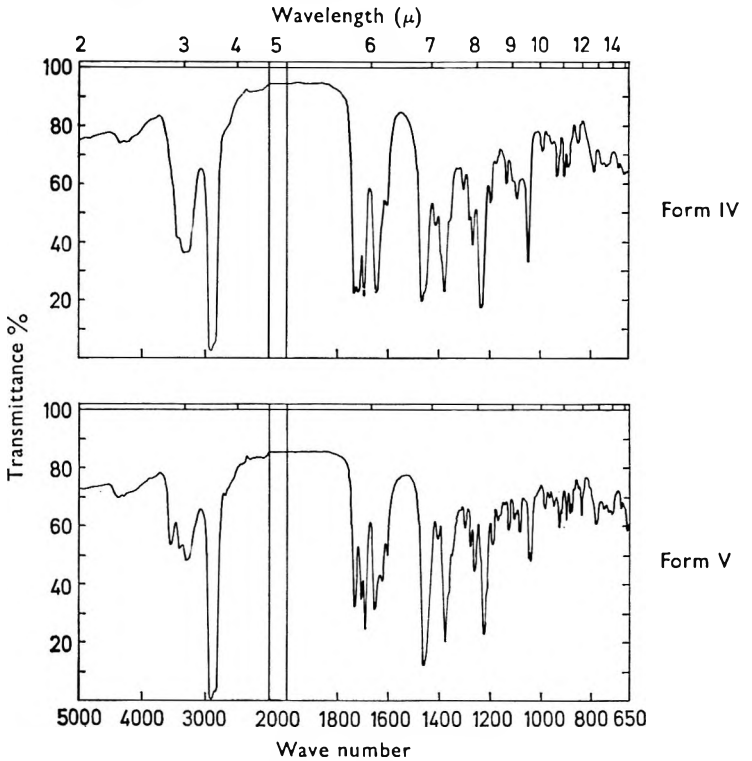

FIG. 1 (continued). Infrared spectra of cortisone acetate crystal forms in Nujol mulls.

STABILITY AND INTERCONVERSION OF THE DIFFERENT FORMS

Forms II–V can be prepared from any form of cortisone acetate by crystallising from the appropriate solvent as described under 'Preparation of different forms.' The final crystal form was independent of the original form.

The different forms were ground in an agate mortar for periods up to 45 min; grinding was also carried out in an agate ball mill (Glen Creston—Model M.270) for periods up to 15 min. Such treatment had little effect on the crystal form.

When an aqueous suspension of *any* form was stored (2 weeks) at room temperature, it underwent a change to Form IV (an extra band at 870 cm^{-1} appeared in the infrared spectrum). Only in the transformation of Form II was Form III detected as an intermediate. Continuous grinding of all forms (except Form IV) under water for 45 min in an agate mortar produced Form III. When an aqueous suspension of Form II was maintained at 100° for 30 min it showed no change in form but an increased number of steroid impurities were detected by chromatography.

If heated to 200° all forms change rapidly to Form I without an increase in the number or amount of related steroid impurities. Prolonged heating at lower temperatures favours the change from Form V to III to II and finally to I. The last stage in that sequence is particularly slow.

A summary of the course of interconversions involved under various conditions is illustrated diagrammatically in Fig. 5.

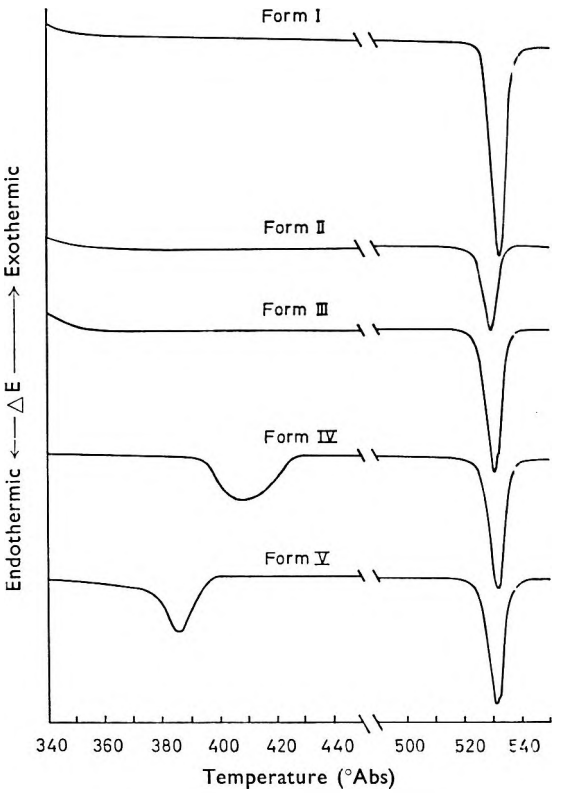

FIG. 2. Thermal analysis curves of cortisone acetate crystal forms. (△E is proportional to millicalories per sec).

Discussion and conclusions

Cortisone acetate in the solid state exists in one of five different crystalline modifications; Forms I, II and III are anhydrous; Forms IV and V are hydrated. In defining the stability of any one of these, it is important to specify the storage conditions used. The three anhydrous forms show true polymorphic behaviour, the other two represent different degrees of hydration, probably of one or other of the anhydrous forms. The way in which water molecules are linked to cortisone acetate molecules is still obscure, although the relatively high energy required for their release suggests some form of bonding to the cortisone acetate molecules.

Callow & Kennard (1961) described methods for the preparation of the different forms of cortisone acetate, but in the present investigation

it was found difficult to reproduce all their results. In some cases the solvent composition, and in particular the water content, are critical. For Form III, the acetone must contain *some* water (>10%), otherwise mixtures of various forms tend to be produced. In case of Form V, methanol must be anhydrous; ~2% water content produced Form IV instead.

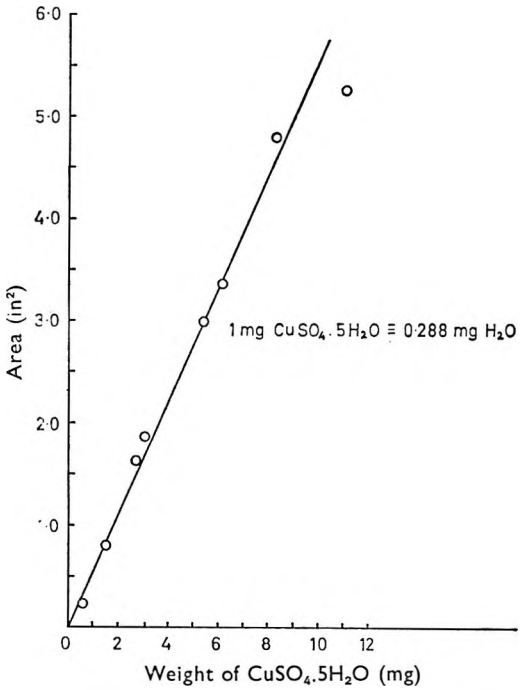

FIG. 3. Differential scanning calorimeter effluent analyser calibration curve for water vapour (scanning speed = 32° per min). ($CuSO_4 \cdot 5H_2O \rightarrow CuSO_4 \cdot H_2O + 4H_2O$).

In general, crystallisation from non-polar solvents gives rise to anhydrous forms (Form II in most cases). On the other hand, polar solvents lead to different forms depending mainly on the water content of the system. Unexpectedly, solvents containing too much water favoured the formation of anhydrous forms (Form III in most cases), whereas very *slightly* hydrated solvents (<1% water) gave rise to highly hydrated forms. Difficulties in preparing any of these forms often arise because the above factors are overlooked. It has been calculated from elemental analysis and loss of weight at 140°/15 mm (Callow & Kennard, 1961) that Form IV contains two molecules of water. Our evidence shows that only half a molecule of water is associated with each molecule of cortisone acetate.

For the preparation of stable aqueous suspensions, Form IV appears to be most suitable. This conflicts with Callow & Kennard (1961), who reported that Form III was the stable form.

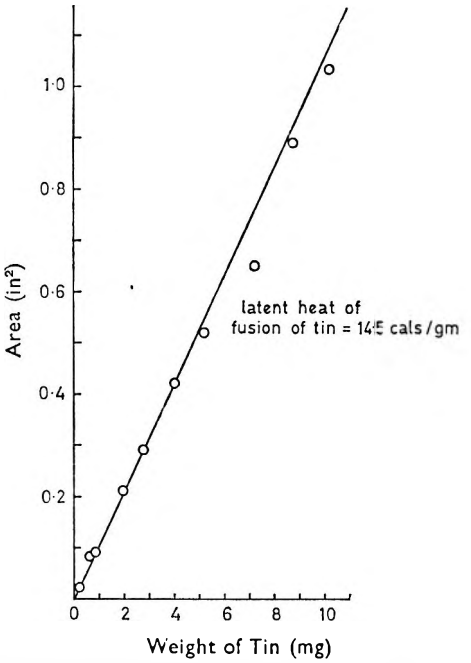

FIG. 4. Differential scanning calorimeter calibration curve for energy uptake (scanning speed = 32° per min).

High temperature treatment causes a decrease in the water content, then produces the thermally stable Form I which resists further heating up to its melting point. The constancy of the amount of energy required for melting supports the view that all forms change to Form I before melting. The transformations mentioned above still take place at room temperature, but at a much slower rate. The kinetics of these transformations are being studied and the work is still in progress.

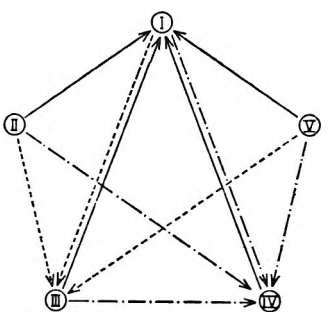

FIG. 5. Interconversion of different cortisone acetate forms. ———heating to 200°. -------grinding under water. — · — · — · — suspension in water.

Form V is very labile to heat and water treatments. The first sign of transformation being loss of its enol band (3540 cm^{-1}) from the infrared spectrum.

The infrared spectrum of Form I may be regarded as definitive for identification purposes. Nujol mulls are preferable because of the sharpness of the bands produced, in comparison to the blunt bands seen in the spectrum of a halide disc. The high pressure applied during the preparation of a disc brings about a partial change of form and this is particularly seen in the hydroxyl stretching region.

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Rheological investigation of a thixotropic lotion

M. WOODMAN AND ANN MARSDEN

Various methods of characterising the thixotropic properties of an oil in water lotion are described. Stress relaxation behaviour, which can be expressed over a range of shear rates by a single empirical equation, is shown to be more informative than the measurement of hysteresis loops. Relaxation rate depends upon previous shear treatment which suggests that more than one process occurs during breakdown.

AN important aspect of quality control of many fluid pharmaceutical preparations is the determination of their flow properties. Such measurements may be correlated with subjective assessments of consistency and in some cases with physical stability. For non-Newtonian materials it is desirable that the flow properties be investigated before an attempt is made to devise a routine testing procedure. With this object the present study was made on a corticosteroid lotion base consisting of a thixotropic aqueous dispersion of cetostearyl alcohol.

Thixotropic systems exhibit stress relaxation when subjected to a steady shear rate and afterwards recover their initial state relatively slowly. The phenomenon is considered to be due to the presence of a shear-sensitive structure containing interparticle or intermolecular links (Hauser & Reed, 1937; Goodeve, 1939; Voet, 1947). The higher the shear rate, the quicker and more pronounced the breakdown. Hysteresis is therefore observed when data obtained at increasing and decreasing rates of shear, the "upcurve" and "downcurve", are compared.

Stresses measured in a thixotropic system must be related to a particular initial structural state and to the procedures used for varying the rate of shear with time. Of the countless possible methods of making such experiments certain logical sequences facilitate the presentation and interpretation of results. Procedures used by various workers include production of hysteresis loops (Green & Weltmann, 1943, 1946; Weltmann, 1943; de Butts, Hudy & Elliot, 1957; Foernzler, Martin & Banker, 1960), measurements of stress relaxation (Hauser & Reed, 1937; Weltmann, 1943; Carver & Van Wazer, 1947) or recovery (Hauser & Reed, 1937; de Waele, 1961; Levy, 1962) and the comparison of equilibrium and initial stresses at various shear rates (Van Wazer, Lyons, Kim & Colwell, 1963). In interpreting the results two effects must be separated, namely the relaxation or recovery of stress with time at constant shear rate, and the change in stress produced by a change of shear rate only. Measurement of stress relaxation in individual samples at various shear rates is an ideal although tedious procedure. In the present work this method has been modified to obtain the same information from one sample and the results compared with those using the hysteresis loop technique.

Experimental

The composition of the lotion was (%w/w): cetostearyl alcohol, 3·0; cetomacrogol 1,000, 0·6; methyl hydroxybenzoate, 0·15; propyl hydroxy-

From the Pharmaceuticals Division, Imperial Chemical Industries Ltd., Macclesfield, Cheshire.

benzoate, 0·08; propylene glycol, 5·0; citric acid, 0·01; and water to 100·0 (all ingredients of B.P. or B.P.C. grade). Samples were prepared as follows: The cetostearyl alcohol, cetomacrogol 1000 and propyl hydroxybenzoate were emulsified at 70° with an equal weight of a solution of the methyl hydroxybenzoate in 85 parts of water. This concentrated emulsion was diluted with the remainder of the aqueous solution at 70°. After cooling to 50° the propylene glycol and citric acid dissolved in sufficient water to make the required weight were added. The lotion was allowed to cool undisturbed to ambient temperature: no homogenisation was necessary. The product was a translucent viscous lotion which was shown by microscopic examination to consist of a fine dispersion of solid cetostearyl alcohol. Measurements were made at 25° on two batches using the Haake "Rotovisco" and Contraves Epprecht "Rheomat" viscometers with measuring systems MVI and B respectively. Shear rates were calculated from instrument dimensions and the speed of rotation and were therefore strictly "apparent shear rates" applicable only to Newtonian fluids. True shear rates at the measuring bob surface would in all instances be higher due to the non-Newtonian flow properties of the lotion. Samples were introduced with as little disturbance as possible and allowed to stand overnight before determining stress relaxation rates, a separate sample being used for each shear rate. Hysteresis loop measurements were made after standing for only 10 min using the method of Green & Weltmann (1943, 1946), keeping the time "t" at which readings were taken proportional to the speed of rotation and hence the shear rate, i.e. $t = \rho \times \text{rpm}$ where ρ is a constant. Recovery of structure after shear was too slow for convenient measurement in the concentric cylinder viscometers. However the reversible nature of the breakdown was confirmed in a separate experiment using the Brookfield Syncrolectric viscometer Model LVT with Helipath accessories.

Results and discussion

The stress relaxation data plotted in Figs 1 and 2 show the relationship between stress and time at each shear rate to be represented over a wide range of time by the empirical equation;

$$\log\left(\frac{\tau_a}{\tau_b}\right) = K \log\left(\frac{t_b}{t_a}\right) \quad .. \quad .. \quad .. \quad (1)$$

where τ_a and τ_b are the values of stress at times t_a and t_b. Values of K have been calculated for each line and are listed in Table 1. For shear rates below 200 sec^{-1} K is virtually constant for each batch of lotion. The lower values of K at high shear rates may be due to a change in true shear rate with time as the viscosity falls, or to the onset of curvature. The Rotovisco results exhibit more scatter particularly at the lowest shear rates due to unsteadiness in the readings. It is interesting to note that although stress recovery after shear has been similarly represented by de Waele (1961) for thixotropic paints, stress relaxation behaviour has been differently interpreted by other workers. Thus Weltmann (1943) found that her data on printing inks gave straight lines when plastic

TABLE 1. VALUES OF K FOR LOTIONS A AND B AT VARIOUS SHEAR RATES

Lotion	Instrument	Apparent shear rate, sec^{-1}	K
A	Rotovisco	571	0·122
,,	,,	347	0·133
,,	,,	190	0·155
,,	,,	127	0·162
,,	,,	63·4	0·166
,,	,,	21·2	0·164
,,	,,	7·05	0·164
B	Rheomat	196	0·195
,,	,,	84·5	0·202
,,	,,	32·9	0·195
,,	,,	9·67	0·189
,,	,,	3·11	0·187

viscosity, U, was plotted against log t. Since $U = (\tau - f)/\sigma$ and f the so-called "yield value" was taken as constant for constant shear rate, σ, it follows that $d\tau/d \log t = a$ constant. Hahn, Ree & Eyring (1959) have contested this relationship and pointed out that the plots were sigmoidal. They have shown from theoretical considerations that stress relaxation should be given by $\log (\tau - \tau_\epsilon) = p - at$, where τ_ϵ is the equilibrium stress, τ = stress at time t and p and a are material constants. Van Wazer & others (1963) have expressed the data of Carver & Van Wazer (1947) on a thixotropic emulsion in a similar way.

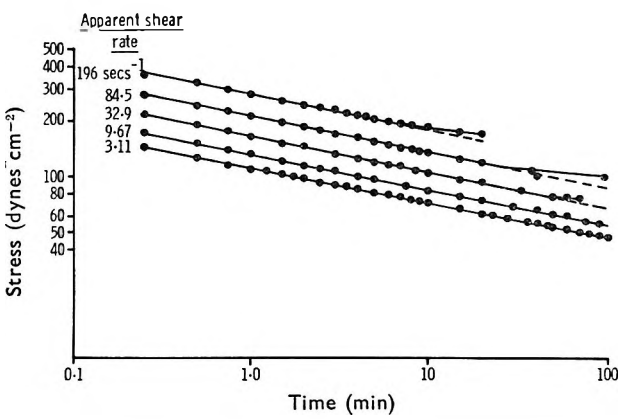

FIG. 1. Stress relaxation at constant shear rates using Rheomat viscometer.

However, from the results in Figs 1 and 2 the value of τ_e is uncertain even at the highest shear rates used for the present system; at the lowest shear rates studied no estimate can be made even after 2 hr. This type of relaxation behaviour has the important result that it is not possible to describe the relationship between stress and shear rate in terms of either initial or equilibrium conditions. A practical alternative is to plot stress (after shearing the sample for some fixed time) *versus* shear rate. Strictly, a fresh sample should be used for each determination, but an attempt was

made to overcome this requirement by making an experiment in which shear rate was progressively raised, allowance being made at each step for the loss of structure already produced.

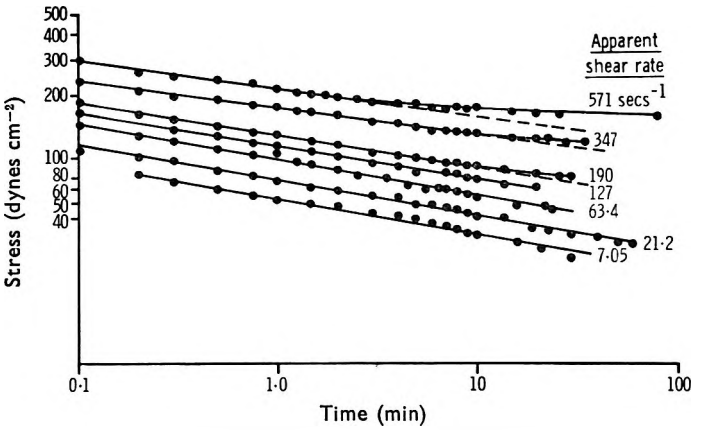

FIG. 2. Stress relaxation at constant shear rates using Rotovisco viscometer.

When stress relaxation measurements are made on samples which have already been sheared at a lower shear rate the stress values are lower than for fresh samples. To determine from measurements on a previously sheared sample how a fresh sample would behave some correction must therefore be applied. It may be considered that the breakdown in structure occurring at the lower shear rate could equally have been produced by shearing for a shorter time at the higher rate; the value of this time must be calculated and added as a correction. If the shear-induced loss of structure be denoted by Δ, and considered to be some function of the amount of shear, i.e.

$$\Delta = f(\sigma t) \qquad (2)$$

it follows that if

$$\Delta(\sigma_1, t_1) = \Delta(\sigma_2, t_2)$$

then

$$t_2 = t_1\sigma_1/\sigma_2 \qquad (3)$$

Data obtained at shear rate σ_2 after shearing for time t_1 at σ_1, where $\sigma_2 > \sigma_1$, should become identical with data on a fresh sample if an appropriate quantity, t_2, is added to each value of t. This method of correction has been applied in Fig. 3 to data obtained on a sample sheared for 140 min at 3·11 sec^{-1} before switching to 9·67 sec^{-1}. By equation (3)

$$t_2 = 140 \times 3{\cdot}11/9{\cdot}67 = 45 \text{ min.}$$

Only the three points obtained during the first 2 min after changing the shear rate are more than 5% from the line. Probably during this time some structure is broken down which can only be removed at the higher shear rate. Similar corrections applied to a sample sheared for 5 min at

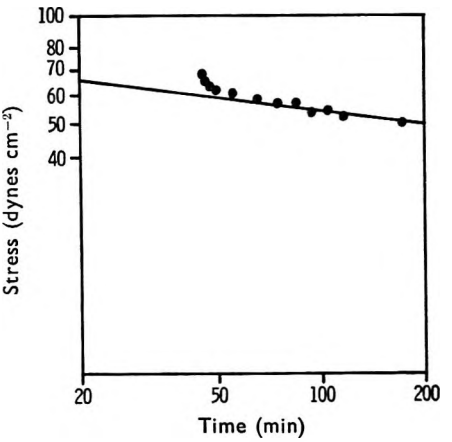

FIG. 3. Stress relaxation after previous shear at a lower rate using Rheomat viscometer. Solid line is relaxation rate of a fresh sample at 9·67 sec^{-1}. Experimental points have been corrected for previous shear at 3·11 sec^{-1}.

5 shear rates from 3·11 to 196 sec^{-1} were equally successful, indicating equation (2) to be a useful first approximation.

Equation (3) can be used to calculate time intervals for an upcurve giving constant equivalent time at each shear rate. Table 2 lists a typical procedure for the Rheomat viscometer. In Fig. 4 such an upcurve is presented for intervals equivalent to 2 min at each shear rate. Five values determined on fresh samples are included for comparison. More

TABLE 2. PROCEDURE FOR OBTAINING AN UPCURVE UNDER CONDITIONS EQUIVALENT TO 2 MIN SHEAR AT EACH SPEED USING SINGLE SAMPLE

Speed	Apparent shear rate (sec^{-1}	t_s (from eqn 3) = time equivalent to 2 min at next lower speed (min)	$2 - t_s$ (min)
1	3·11	0	2
2	4·18	2 × 3·11/4·18 = 1·49	0·51
3	5·50	2 × 4·18/5·50 = 1·52	0·48
4	7·33	1·50	0·50
5	9·67	1·52	0·48
6	13·9	1·39	0·61
7	18·7	1·49	0·51
8	24·6	1·52	0·48
9	32·9	1·50	0·50
10	43·4	1·52	0·48
11	62·9	1·38	0·62
12	84·8	1·48	0·52
13	111	1·53	0·47
14	148	1·50	0·50
15	196	1·51	0·49

information is imparted by a curve of this type supplemented by a stress relaxation plot than by hysteresis loops, examples of which are given in Fig. 5. The upcurves are distorted because the equivalent time at each shear rate is not uniform when ρ is maintained constant.

Equations for the flow curves other than in empirical terms do not seem to be possible. Most theoretically derived equations depend upon

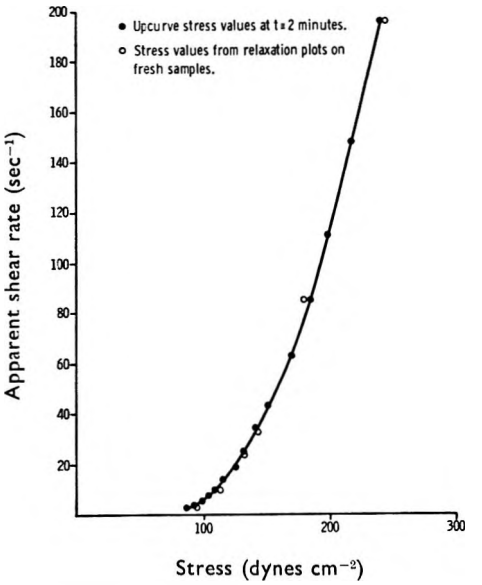

FIG. 4. Upcurve using Rheomat viscometer. Stress values were obtained under conditions equivalent to constant time at each shear rate.

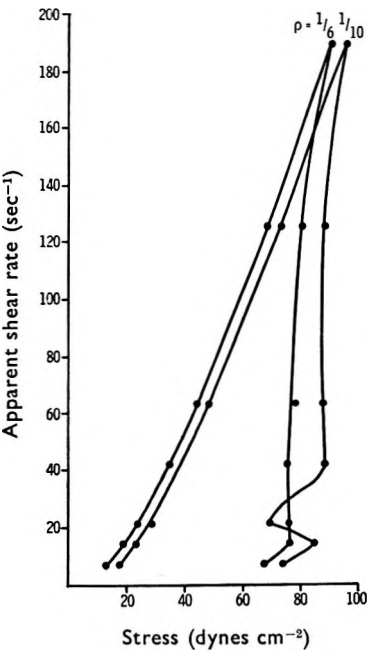

FIG. 5. Hysteresis loops obtained using Rotovisco viscometer. For explanation of ρ see text.

steady state conditions being reached (Goodeve & Whitfield, 1938; Powell & Eyring, 1944; Casson, 1959; Gillespie, 1960a, b; Cross, 1965) whilst Green & Weltmann's upcurve equation (1943) can apply only if loss of stress is proportional to shear rate. The equation of Hahn & others (1959) for non-equilibrium conditions is applicable to simple shear-promoted transitions such as molecular disentanglement but in the present systems there is evidence that more than one process of breakdown, each with a different shear rate dependency, is involved. Thus on going from a high to a much lower shear rate there is a short period of rapid stress recovery and similarly on returning to the high shear rate some recovery is found to have occurred. Breakdown then occurs much more quickly than on the fresh sample until the point is reached again at which the high shear rate was initially interrupted. We concur with Weltmann (1960) that some structure can be broken only at high shear rates.

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Water vapour transmission properties of applied polymer films

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The rates of moisture uptake by film-coated tablet matrices containing calcium chloride have been determined as a function of polymer coat formulation, film coat thickness and calcium chloride content of the matrix. The more hydrophilic polymer film formulations were less effective in reducing the rate of moisture uptake by the tablet matrices. A linear relation was observed between rate of moisture uptake and film thickness, in contrast to the log-log relationship obtained in previous work using the same systems cast as free films. Decreasing the calcium chloride content of the matrix caused an overall lowering of the moisture uptake rate. The results indicate that the water vapour transmission characteristics of these polymer film systems are not the same when assessed using the rate of moisture uptake of tablets coated with these films as when using the rate of water vapour transmission through the free films. It is suggested that films should not be accepted or rejected solely on the basis of transmission rates across free films but that due consideration should be given to the more meaningful moisture uptake rates of the film-coated dosage form.

THERE are several reports about the permeability of free polymer films with potential as film coating materials (see Banker, Gore & Swarbrick, 1966). No report dealing with the permeability properties of such films when applied to hygroscopic solid matrices appears to have been made nor any attempt to correlate the water vapour transmission properties of free and applied polymer films.

Banker & others (1966) reported the water vapour transmission properties of a hydrophilic, a lipophilic and a mixed hydrophilic-lipophilic polymeric film-forming system cast as free films. Preliminary investigations on these same systems when applied to tablet matrices of varying hygroscopicity are now reported.

Experimental

PREPARATION OF TABLET MATRICES

Table 1 summarises the various tablet matrices prepared and the compositions of the film coatings applied. Matrix A was prepared by dry slugging. It was necessary to granulate the microcrystalline cellulose used in matrices B and C with 10% w/v polyvinylpyrrolidone in isopropanol. The dicalcium phosphate dihydrate used in matrices D and E was granulated with a warm aqueous solution of gelatin (12% w/v) and acacia (4% w/v). Magnesium stearate (2% w/w) was used as a lubricant throughout. A Stokes rotary tablet press (model 512-1) fitted with standard 7/16 inch concave punches was used to prepare the tablets which had an average weight of 650 $\pm$ 12 mg and a hardness of 8 $\pm$ 1 kg when measured

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with a Pfizer tablet hardness tester. The tablets were stored in tightly closed glass containers at room temperature in the presence of silica gel until required.

TABLE 1. FILM COATED SYSTEMS STUDIED

Matrix	Composition of Matrix (% w/w)		Film coats applied
A	Microcrystalline cellulose[1]	50	HPC[2]; MHPC:EC[3]; BMA[4]
	Calcium chloride	50	
B	Microcrystalline cellulose	70	HPC
	Calcium chloride	30	
C	Microcrystalline cellulose	80	HPC
	Calcium chloride	20	
D	Dicalcium phosphate dihydrate	95	HPC; MHPC:EC; BMA
	Calcium chloride	5	
E	Dicalcium phosphate dihydrate	100	MHPC:EC

[1] Avicel, FMC Corporation, American Viscose Division, Newark, Delaware, U.S.A.
[2] Hydroxypropyl cellulose plus 20% propylene glycol, used as a 5% w/v polymer solution.
[3] Methylhydroxypropyl cellulose:ethyl cellulose (3:1) plus 50% propylene glycol, used as a 3% w/v polymer solution.
[4] n-Butyl methacrylate plus 5% diethyl phthalate, used as a 10% w/v polymer solution.

FILM COATING OF TABLETS

A Binks airless spray coating system operating at a pressure of 50 psi was used. The spray nozzle had an opening of 0·011 in and a spraying angle of 40°. One thousand tablets were placed in a coating pan (diameter 7·5 in) rotating at 65 rpm. The tablets were warmed with a hot air blower and an exhaust hose was used to remove solvent vapours. The temperature within the pan was maintained at 45–50° and the humidity within the coating room at 20–25% relative humidity. The tablets were sprayed with from 1 to 5 sec bursts of the film coat solution, sufficient time being allowed between each spray to permit evaporation of solvent and formation of the film. The total number of sprays given any one batch varied depending on the concentration of the polymer-plasticiser combination in the spraying solution, which in turn depended on the viscosity of the polymer in solution. Tablets were removed at regular intervals and stored in tightly closed glass containers at room temperature in the presence of silica gel. Measurements of film coat thickness were made with a micrometer on the peeled film coat as well as on the coated and uncoated tablets.

DETERMINATION OF MOISTURE UPTAKE

The moisture uptake cell consisted of a cylindrical screw capped bottle, capacity approximately 23 ml, containing 5 ml of a saturated sodium tartrate solution which maintained a vapour pressure of 29·0 mm Hg at 30° (Lowry & Morgan, 1924). The tablet was suspended within the cell by a wire loop which pierced the cap liner. The moisture uptake of both coated and uncoated tablets was determined by removing and weighing the cap plus tablet at intervals over 12 hr. It had been determined previously that the cap and wax liner did not change weight under the conditions of the experiment.

To assess the moisture uptake of the film coat, blank tablets of dicalcium phosphate dihydrate containing no calcium chloride (matrix E) were film

coated with the mixed polymer formulation along with tablets of matrix D. The tablets of matrix E contained amaranth dye (1 : 1500 parts by weight) to permit their ready separation following film coating from those containing calcium chloride. Moisture uptake of the uncoated blank tablets of matrix E was also measured.

Results and discussion

The change in weight of the uncoated tablet matrices A, B and C increased linearly with time over the period studied. With the uncoated

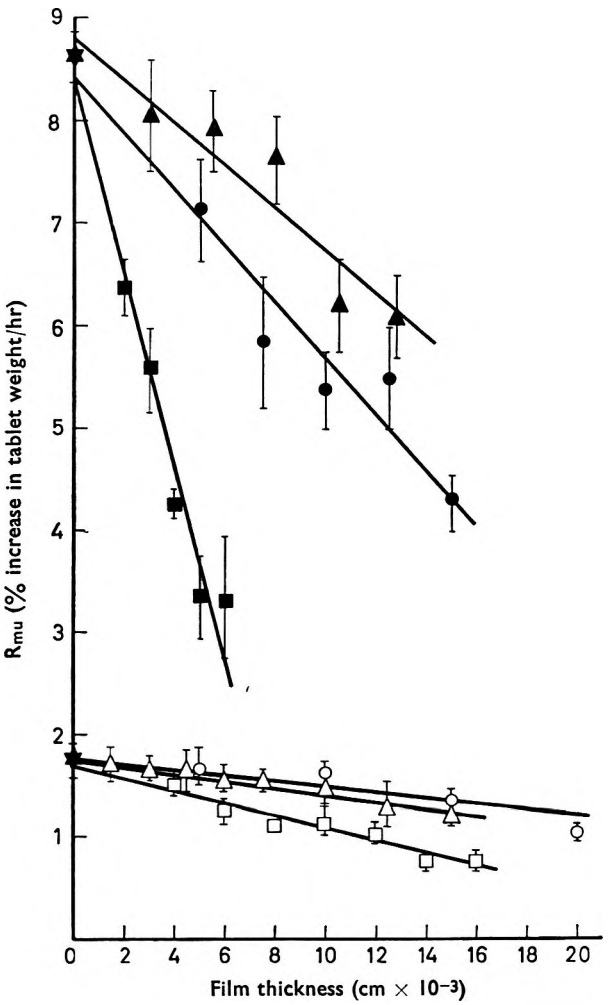

FIG. 1. Effect of film coat thickness on the rate of moisture uptake of tablet matrices containing 5 and 50% calcium chloride at 30°. Uncoated tablets, ★; n-butyl-methacrylate: matrix A, ■; matrix D, □; hydroxypropyl cellulose: matrix A, ●; matrix D, ○; methylhydroxypropyl cellulose: ethyl cellulose: matrix A, ▲; matrix D, △.

matrix D the rate became linear after 2–3 hr. The moisture uptake by the tablets film-coated with n-butyl methacrylate was linear with time and no macroscopic changes in the film coat were apparent even though matrix A, containing 50% calcium chloride, did swell noticeably. The possible effect of the resultant stretching of the film upon its permeability was therefore held to be insignificant. Initial uptake of moisture by the

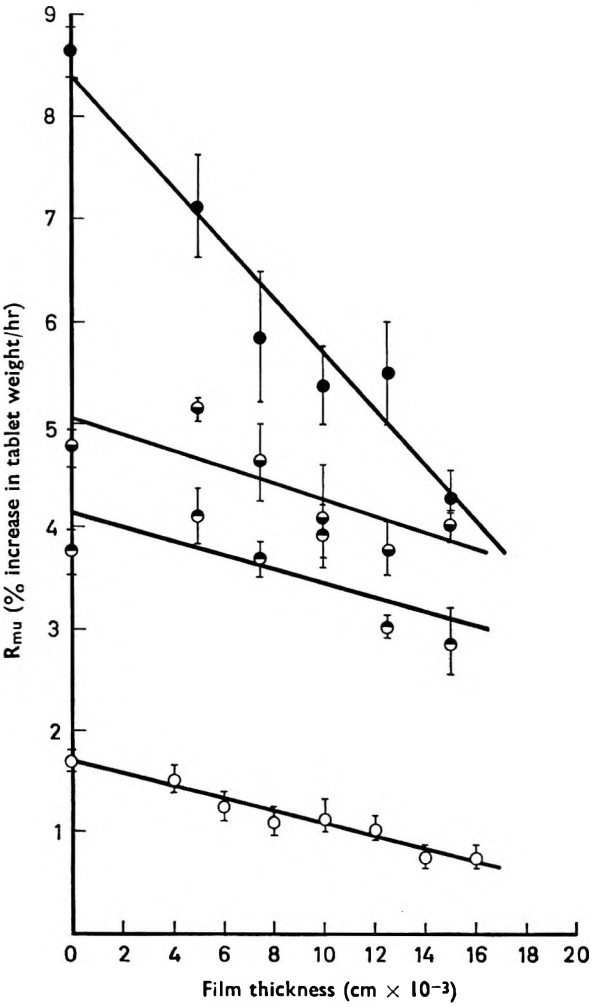

FIG. 2. Effect of film coat thickness on the rate of moisture uptake of tablet matrices containing 5, 20, 30 and 50% calcium chloride at 30°. Hydroxypropyl cellulose: matrix A, ●; matrix B, ◓; matrix C, ◒; matrix D, ○.

hydroxypropyl cellulose film-coated tablets was also linear with time using matrices A, B and C. However, the swelling of the core caused the coat to break after approximately 6 hr, whereupon moisture uptake was accelerated. With matrix D the rate was constant from 2 to 5 hr and no

rupturing of the film was observed. With the mixed polymer coat applied to matrix A, the films became noticeably mucilaginous and thickened after 4 hr in the moisture uptake cell, due presumably to partial solution of the methylcellulose. The films did not rupture and the rate of moisture uptake decreased after this point. This phenomenon was not observed with film-coated tablets of matrix D. It is worth noting that the various films did not exhibit these changes when used as free films in permeability studies (Banker & others, 1966). This demonstrates the need to observe the performance of polymer films under conditions which closely simulate their actual use.

The total moisture uptake of both the coated and uncoated tablets of matrix E lay within the range 0·9–1·1% of the original tablet weight and was complete within 2–3 hr. The amount of moisture absorbed by the film coat was therefore negligible compared to that absorbed by the matrices containing calcium chloride, a result not unexpected in view of the volume of film coat applied (Banker & others, 1966). Since the moisture uptake of the blank matrix was complete within 3 hr, and in the light of the previous discussion concerned with changes in the integrity of the film after 4 or more hours, the rate of moisture uptake at 3 hr was chosen in all instances.

Fig. 1 shows the rate of moisture uptake by matrices A and D uncoated and also when film coated with the three polymer-plasticiser formulations. The effect of calcium chloride content upon the moisture uptake rate for hydroxypropyl cellulose coated tablets is shown in Fig. 2. In both figures each point represents the mean of at least 2 determinations; the vertical lines represent the range of values obtained. The rate is expressed as the percentage increase in weight with respect to the average uncoated tablet weight.

The relation between the rate of moisture uptake and film thickness, illustrated in Figs 1 and 2, may be expressed as follows:

$$R_{mu} = mt + C \qquad (1)$$

where R_{mu} is the percentage increase in the original average tablet weight per hr, t the film thickness in cm, m the slope of the line and C the ordinate intercept. The statistical constants for these plots are presented in Table 2. In view of the number of possible sources of error in the manufacture and film coating of the tablet matrices, the correlation is held to be good.

TABLE 2. STATISTICAL PARAMETERS FOR MOISTURE UPTAKE OF FORMULATIONS STUDIED

Film system	Matrix	Correlation coefficient	Slope of line m	Ordinate intercept C
n-Butylmethacrylate + 5% diethyl phthalate	D	0·97	−609	1·69
	A	0·98	−936	8·37
Hydroxypropyl cellulose + 20% propylene glycol	D	0·96	−32·1	1·83
	C	0·72	−68·1	4·12
	B	0·83	−78·8	5·05
	A	0·97	−275	8·42
Methylhydroxypropyl cellulose : ethyl cellulose (3 : 1) + 50% propylene glycol	D	0·97	−35·1	1·76
	A	0·94	−209	8·82

The effect of film formulation on moisture uptake is only qualitatively similar to that observed in water vapour transmission studies on the free films. Thus tablets containing 50% calcium chloride and coated with the hydroxypropyl cellulose and mixed polymer film formulations show a higher rate of moisture uptake than those coated with the same thickness of the n-butyl methacrylate film. Similar results were observed with tablets containing 5% calcium chloride. The decrease in R_{mu} per unit increase in film thickness is again greatest in those tablet matrices coated with the lipophilic n-butyl methacrylate film.

The main point of difference between the moisture permeability of the free and applied films is in the relation between film thickness and the rate of permeation or uptake. With the free films, the rate decreased linearly with thickness only when these parameters were expressed on a log-log basis. With the applied films the rate of moisture uptake was a direct function of the film thickness. One possible explanation is that the affinity of the tablet matrix for moisture is such that Fickian, or near-Fickian, diffusion through the film is supplanted by the chemical affinity of the substrate for moisture immediately adjacent to the distal surface of the film. Work is currently in progress to establish whether or not this phenomenon is common to all hygroscopic film coated matrices.

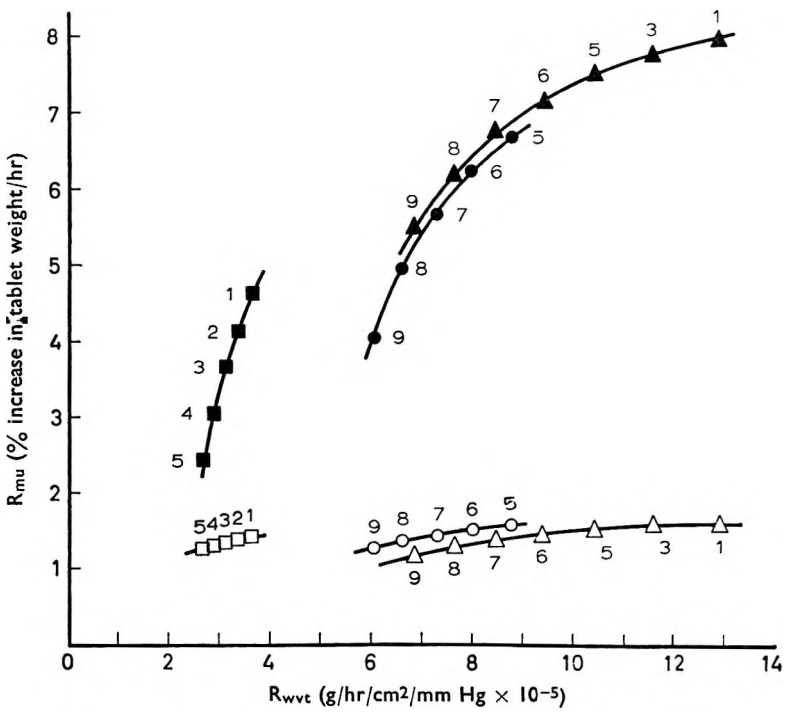

FIG. 3. Relationship between rate of moisture uptake of film coated tablet matrices and water vapour transmission rate of free polymer films at 30°. Key. Polymer systems as in Fig. 1; film coat thickness (cm × 10^{-3}); 1, 4·0; 2, 4·5; 3, 5·0; 4, 5·6; 5, 6·3; 6, 8·0; 7, 10·0; 8, 12·6; 9, 15·9.

The results of this preliminary study establish the existence of a "*within film system*" *correlation* between the water vapour transmission properties of polymers when cast as free and applied films. Thus, we have plotted the water vapour transmission rates (R_{wvt}) presented previously (Banker & others, 1966) against the moisture uptake rates of the various film coated systems at equivalent film thicknesses (Fig. 3). It is apparent, however, that the various film systems which show identical R_{wvt} values do not confer identical R_{mu} values upon the coated dosage form. Furthermore, as shown in Table 3, the ratio of R_{mu} to R_{wvt} at a particular film

TABLE 3. RATIO OF THE PERCENTAGE INCREASE IN THE ORIGINAL AVERAGE TABLET WEIGHT PER HOUR R_{mu} TO R_{wvt}, THE WATER VAPOUR TRANSMISSION RATE, FOR FREE FILMS, 6·30 cm × 10^{-3} THICK AT 30°

Film system	$(R_{mu}/R_{wvt}) \times 10^3$	
	Matrix A	Matrix D
n-Butyl methacrylate	0·92	0·48
Hydroxypropyl cellulose	0·76	0·18
Methylhydroxypropyl cellulose : ethyl cellulose (3 : 1)	0·72	0·15

thickness for a polymer-matrix system is not a constant between films and the same matrix. The effect of reducing the calcium chloride content of the matrix is also not constant between the polymer systems investigated. Consequently, it would appear a worthwhile practice at this time not to accept or reject a film coat formulation solely on the results obtained using water vapour transmission across the free film but to evaluate the performance of the film when applied to the particular dosage form in question.

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