RESEARCH PAPERS

THE EFFECT OF *ORTHO* SUBSTITUTION ON THE HYDROLYSIS OF BENZOYLCHOLINE

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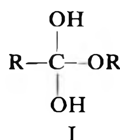
A series of mono- and di-*ortho*-substituted benzoylcholine compounds has been prepared and the rates of hydroxide ion and cholinesterase catalysed hydrolysis has been determined for each compound. The anti-acetylcholinesterase activities of the compounds have also been determined. It has been found that some of the compounds are stable to esterase catalysed hydrolysis and that the groups which confer stability do not prevent the formation of enzyme substrate complexes. The most suitable groups for ester stabilisation, when used as *ortho* substituents, are alkyl and iodo as mono substituents and most groups, except hydroxy, as di-*ortho* substituents.

THE *in vivo* hydrolysis of benzoyl ester groups is a limiting factor in the use of some drugs and consequently it would be of value to stabilise such esters. The hydrolysis of benzoylcholine is catalysed both by hydroxide ions and by serum cholinesterase and therefore this molecule is suitable to examine the influence of *ortho* substitution on esterase catalysed hydrolysis of benzoyl esters. Ormerod (1953) prepared several *meta* and *para* substituted benzoylcholine compounds and determined their rates of cholinesterase catalysed hydrolysis. The small differences observed between experimental results and results calculated using the Hammett substituent constant were attributed to "differences in the enzyme substrate complex".

Ortho substituents in benzoate esters have been shown (Evans, Gordon and Watson, 1937; Ingold, 1953a) to accelerate less or retard more the rate of ester hydrolysis than the same substituents in the *meta* and *para* positions. Consequently, *ortho* substitution of benzoylcholine may produce large changes in the rates of cholinesterase catalysed hydrolysis.

The interactions between an ester group and an *ortho* substituent may be direct or transmitted or a combination of both these effects. The direct effect is exerted by way of electrostatic forces, van der Waal's forces and short range repulsive forces. The effects of the latter forces have been termed the primary steric effect (Ingold, 1953b). Transmitted interactions are due to induction and mesomerism. Where steric repulsion alters the geometry of a molecule, thereby modifying the transmitted interactions, the effect has been termed the secondary steric effect (Ingold, 1953c).

Bender (1951) demonstrated the presence of a tetrahedral intermediate of the type I in the acid and alkali catalysed hydrolysis of esters.



The attack of an hydroxide ion to form this intermediate is said to be from a direction perpendicular to the plane of the ester group (Brown and Fried, 1943; Kadesch, 1944). If this plane is coplanar with the benzene ring then the *ortho* groups do not sterically prevent this approach (primary steric effect). *Ortho* groups may prevent free rotation of the carboxyl group around the bond connecting this group to the ring and may prevent coplanarity of the ester group and the benzene ring (secondary steric effect). This would have the effect of a perpendicular approach being hindered by the *ortho* substituents. One sufficiently large *ortho* group can prevent coplanarity of the ester group with the ring. Reaction is still possible but only from the side opposite to the hindering substituent.

When only one *ortho* substituent is present the direct effects are additional to those of induction and mesomerism. However, when two *ortho* substituents are present the direct effects completely dominate the transmitted interactions and most di-*ortho* substituted benzoyl esters are stable to hydrolysis.

The mechanism of esterase catalysed hydrolysis is considered to be initiated by an attack of a site of high electron density of the enzyme on the acyl carbon of the ester (Bergman, Nachmansohn and Wilson, 1950; Davies and Green, 1958; Rydon, 1958), not unlike the attack of basic reagents such as the hydroxide ion. It appears possible therefore, that *ortho* substituted benzoyl esters will be stable to esterase catalysed hydrolysis.

A series of mono- and di-*ortho* substituted benzoylcholine esters has been prepared and the rates of hydroxide ion and cholinesterase catalysed hydrolysis have been determined.

EXPERIMENTAL

Chemical

All the mono substituted benzoic acids were obtained commercially. The preparation of 2-methylbenzoylcholine iodide is typical of the method used for the mono substituted benzoylcholines.

2-Methylbenzoyl chloride. *o*-Toluic acid (25 g.) was refluxed on a steam bath with thionyl chloride (28 g.) until fumes of hydrogen chloride were no longer evolved; about two hr. The solution was distilled under reduced pressure and the fraction b.p. 110° at 29 mm. was collected.

2-Dimethylaminoethyl 2-methylbenzoate hydrochloride. 2-Methylbenzoyl chloride (15·4 g., 0·1 mole) was dissolved in dry benzene (100 ml.). 2-Dimethylaminoethanol (8·9 g., 0·1 mole) was added to the solution which was kept cool during the addition and then refluxed for 30 min. The white solid which precipitated was filtered off and recrystallised from an acetone-ether mixture. The product was a white crystalline solid m.p. 127°. Yield 97 per cent.

2-Methylbenzoylcholine iodide. 2-Dimethylaminoethyl 2-methylbenzoate hydrochloride (15 g., 0·1 mole) was dissolved in water (20 ml.) in a separator. Ammonia solution (7·6 ml., 10 per cent NH_3) was added and the free base completely extracted with chloroform. The chloroform solution was washed with water, dried and methyl iodide (21 g., 0·15

TABLE I

Ortho SUBSTITUTED BENZOYLCHOLINE COMPOUNDS

Compound (*a*) X-Benzoylcholine X=	Acyl halide		Dimethylaminoethyl ester hydrochloride			Choline derivatives			Analysis for choline derivatives					
									Found			Required		
	Reflux time	b.p.	Reflux time	Solv. for recryst.	m.p. °C	Reflux time	Solv. for recryst.	m.p. °C	C	H	I	C	H	I
2-Chloro[c]	(*b*)	110° at 15 m.m.	30 min.	Ethanol Ether	128	30 min.	Ethanol Acetone	174	39·6	4·7	34·4	38·9	4·6	34·4
2-Bromo	(*b*)	125° at 20 mm.	3 hr.	Ethanol Ether	147	2 hr.	Ethanol	142	35·4	4·2	30·6	34·8	4·14	30·7
2-Iodo	2 hr.	159° at 27 mm.	1 hr.	Ethanol Ether	167	1 hr.	Ethanol Ether	152	31·3	3·7	28·0	31·2	3·69	27·6
2-Nitro	2 hr.	Unsafe to distil[d]	15 min.	Ethanol Ether	171	1 hr.	Ethanol Ether	155	38·0	4·5	32·9	37·9	4·51	33·4
2-Methoxy	2 hr.	128° at 11 mm.	2 hr.	Acetone	132	3 hr.	Ethanol	162	42·4	5·5	34·4	42·2	5·48	34·8
2,6-Dimethyl ..	3 hr.	216°	8 hr.	Ethanol	160	1 hr.	Ethanol	168	48·0	6·7	34·8	48·1	6·3	35·0

(*a*) All prepared as iodide salt.
(*b*) Obtained commercially.
(*c*) Ormerod (1953).
(*d*) Bonner and Hurd (1946).

mole) added. The mixture was then refluxed for 2 hr. The precipitated white solid was recrystallised from acetone-ether m.p. 157°. Yield 99 per cent $C_{13}H_{20}INO_2$ requires C, 44·7; H, 5·7; I, 36·39. Found: C, 44·3; H, 5·7; I, 36·2.

A list of ortho substituted benzoylcholine compounds prepared by this method is given in Table I.

2-Dimethylaminoethyl bromide hydrobromide. This was prepared by heating a mixture of 2-dimethylaminoethanol (45 g.) and hydrobromic acid (95 ml., 60 per cent) at 120° in an autoclave for 6 hr. The solution was evaporated to dryness under reduced pressure and the residue recrystallised from ethanol-ether mixture m.p. 173–174° (Huttrer and others 1946; m.p. 187°). Yield 70 g. Found: C, 20·5; H, 4·5; Br, 34·4. Calc. for $C_4H_{11}Br_2N$. C, 20·6; H, 4·7; Br, 34·3.

2,6-Dichlorobenzoic acid. This acid was prepared from 2,6-dichlorotoluene by the method of Norris and Bearse (1940), m.p. 147° (Norris and Bearse, 1940; m.p. 143°). Found: C, 43·82; H, 2·0. Calc. for $C_7H_4Cl_2O_2$; C, 43·9; H, 2·1. (Note. The entire synthesis should be carried out in an efficient fume cupboard since an intensely lachrymatory substance is formed during the preparation.)

Silver 2,6-dichlorobenzoate. 2,6-Dichlorobenzoic acid (9·5 g., 0·05 mole) was added to hot distilled water (100 ml.) to which concentrated ammonia solution (9 ml.) was added. A steam bath was used to remove excess ammonia and a warm solution of silver nitrate (8·5 g., 0·05 mole) was added dropwise with stirring. The silver salt precipitated, was filtered, washed with water and dried at 70° under reduced pressure. Yield 14·8 g.

2-Dimethylaminoethyl 2,6-dichlorobenzoate. Silver 2,6-dichlorobenzoate (6·0 g., 0·02 mole) was finely powdered and added to chloroform (150 ml.). The suspension was stirred, 2-dimethylaminoethyl bromide hydrobromide (4·5 g., 0·02 mole) added and the slurry was refluxed for 48 hr. The hot reaction mixture was filtered under reduced pressure and the solution used for the next stage without isolating the product.

2,6-Dichlorobenzoylcholine iodide. The chloroform solution of 2-dimethylaminoethyl 2,6-dichlorobenzoate hydrobromide was transferred to a separator, ammonia added (5 ml., 10 per cent NH_3) and the chloroform layer separated, washed and dried. Methyl iodide (4 g.) was added to the solution which was refluxed for 1 hr. when the product precipitated. It was filtered, washed and recrystallised from ethanol-acetone mixture m.p. 157°. $C_{12}H_{16}Cl_2INO_2$ requires C, 35·7; H, 3·96; I, 31·43. Found: C, 36·28; H, 4·1; I, 30·7. A list of substituted benzoylcholine compounds prepared by the silver salt method is given in Table II. 2,6-Dihydroxybenzoic acid and 2,4,6-trinitrobenzoic acid were obtained commercially.

2,6-Dimethylbenzoic acid. This was prepared from *m*-2-xylidine by the method of Jacobs and others (1951), m.p. 116° (Jacobs and others, 1951; m.p. 115·5°). Found: C, 72·24; H, 6·6. Calc. for $C_9H_{10}O_2$; C, 72·0; H, 6·7.

2,4,6-Tribromobenzoic acid. This acid was prepared from *m*-aminobenzoic acid by the method of Robinson and Robinson (1956); m.p. 202°

TABLE II

Ortho SUBSTITUTED BENZOYLCHOLINE COMPOUNDS PREPARED BY THE SILVER SALT METHOD

Compound (a) X-Benzoylcholine X=	Silver salt: Quantity of reactants		2-Dimethylaminoethyl HBr ester of: Quantity of reactants			Choline derivatives			Analysis for choline derivatives: Found			Required		
	Acid	$AgNO_3$	Silver salt	Amino halide	Reflux time	Reflux time	Solv. for recryst.	m.p. °C	C	H	I	C	H	I
2,6-Dihydroxy ..	5·0 g. 0·03 mole	5·0 g. 0·03 mole	8·6 g. 0·03 mole	7·0 g. 0·03 mole	48 hr.	30 min.	Ethanol	178	38·5	5·1	—	39·2	4·9	—
2,4,6-Trinitro ..	13·0 g. 0·05 mole	8·5 g. 0·05 mole	8·0 g. 0·02 mole	4·5 g. 0·02 mole	48 hr.	2 hr.	Ethanol	180	30·9	3·4	26·9	30·7	3·2	27·0
2,4,6-Tribromo ..	14·4 g. 0·04 mole	7·0 g. 0·04 mole	13·0 g. 0·03 mole	7·0 g. 0·03 mole	48 hr.	2 hr.	Ethanol Acetone	216	24·5	2·4	22·0	25·2	2·62	22·2

(*a*) All prepared as iodide.

(Robinson and Robinson 1956; m.p. 192·5–1954·5°). Found: C, 23·3; H, 0·8; Br, 65·8. Calc. for $C_7H_3Br_3O_2$: C, 23·4; H, 0·98; Br, 66·8.

Hydrolyses

Enzyme catalysed hydrolysis. An electrically heated, thermostatically controlled water bath, fitted with an electrical stirrer was used. The temperature was adjusted to 37° ± 0·1. A small beaker (100 ml.) was supported in the water bath and into its top was fitted a rubber bung with five holes. Through these five holes were inserted a glass electrode, a glass stirring rod, one arm of an agar bridge, the tip of a microburette, and a small funnel through which solutions were poured. The other arm of the agar bridge dipped into a saturated potassium chloride solution into which also dipped the calomel electrode. Both the glass and the calomel electrodes were connected to a Pye pH meter, the temperature compensator of which was set at 37°.

Horse serum (1 ml.) was pipetted into the funnel and washed into the beaker with distilled water (9 ml.). The pH was adjusted to 7·9 by the addition of approximately 0·01 N sodium hydroxide solution from the microburette. After allowing 15 min. for temperature equilibration the substrate (1×10^{-4} mole), dissolved in distilled water (15 ml.) at 37°, was added through the funnel and washed through with distilled water (5 ml.) at 37°. (The concentration of substrate in the digest was $3{\cdot}3 \times 10^{-3}$ molar.) After 1 to 2 min. 0·01 N sodium hydroxide solution was added until the pH of the solution was again 7·9. The pH was then maintained at 7·9 by the dropwise addition of alkali, the burette readings and the time being recorded. Throughout the determination the solution in the beaker was stirred frequently. At least three hydrolysis experiments were made with each substrate. From the slopes of graphs drawn with ordinates representing ml. of NaOH added and abscissae representing time, the rates of cholinesterase plus hydroxide ion catalysed hydrolysis were calculated. The results, corrected for non-enzymic hydrolysis are given in Table III. The values given are the mean of three measurements. The measured rates of hydrolysis for each compound were all within 5 per cent of the mean.

Source of cholinesterase. Horse serum was used because it has a high content of cholinesterase (Stedman and Stedman, 1935). All the determinations were made using serum from the same commercial batch since differences in cholinesterase activity have been observed with different batches of serum (Buckles, 1956). The serum was stored in a refrigerator at 4°. No deterioration in enzyme activity was observed during the course of the work.

0·01 N *Sodium hydroxide solution.* An approximately 0·2 N stock solution was prepared by dissolving the required amount of A.R. sodium hydroxide in freshly boiled and cooled distilled water. The stock solution was diluted as required and standardised against potassium hydrogen phthalate by a potentiometric titration.

Hydroxide ion catalysed hydrolysis. The rates of hydroxide ion catalysed hydrolysis were measured in a similar way to that used for the

enzymic rates. A dilute buffer solution consisting of 1 ml. of B.P. 1953 boric acid and potassium chloride buffer pH 7·9 was used in place of the serum. At least three hydrolysis experiments were made on each substrate. Graphs were drawn with the ordinates representing ml. of

TABLE III

MEAN RATES, RELATIVE RATES AND THE RATIO OF THE RATES OF CHOLINESTERASE AND HYDROXIDE ION CATALYSED HYDROLYSIS OF *ortho* SUBSTITUTED BENZOYLCHOLINE COMPOUNDS

Compound (*a*) R – Benzoylcholine R=	Rate of cholinesterase catalysed hydrolysis	Rate of hydroxide catalysed hydrolysis	Relative rates of cholinesterase catalysed hydrolysis (*b*)	Relative rates of hydroxide catalysed hydrolysis (*c*)	Ratio: E/NE
	ml. 0·01 N NaOH/min./g. mole				
H	831	156	1·00	1·00	5·30
2-Methyl	203	110	0·24	0·65	1·85
2-Chloro	1,095	219	1·30	1·40	5·00
2-Bromo	580	173	0·70	1·02	3·40
2-Iodo	200	125	0·24	0·74	1·60
2-Nitro	156	237	0·19	1·40	0·65
2-Methoxy	604	120	0·73	0·77	5·00
2,6-Dichloro.. ..	0	0	0	0	0
2,6-Dimethyl ..	0	0	0	0	0
2,6-Dihydroxy ..	Unstable in solution				
2,4,6-Tribromo ..	0	0	0	0	0
2,4,6-Trinitro ..	0	575	0	3·40	0

(*a*) All compounds were used as the iodide salt.
(*b*) Rates relative to cholinesterase catalysed hydrolysis of benzoylcholine = 1.
(*c*) Rates relative to the hydroxide ion catalysed hydrolysis of benzoylcholine = 1.
E Rate of enzymic hydrolysis.
NE Rate of non-enzymic hydrolysis.

NaOH added and abscissae representing time. The rate of non-enzymic hydrolysis was obtained from the slope of each graph. The measured rates of hydrolysis for each compound were all within 5 per cent of the mean. The results obtained are given in Table III.

TABLE IV

ANTI-ACETYLCHOLINESTERASE ACTIVITY OF COMPOUNDS PREPARED. pH 6·3. TEMPERATURE 37°. SUBSTRATE ACETYLCHOLINE, CONCENTRATION 0·003 M. SOURCE OF ENZYME, ERYTHROCYTE STROMATA. I 50 VALUES IN G. MOLES/L.

Compound (*a*)	I 50 value
Benzoylcholine	$2·90 \times 10^{-3}$
2-Methylbenzoylcholine	$9·35 \times 10^{-4}$
2-Chlorobenzoylcholine	$3·29 \times 10^{-4}$
2-Bromobenzoylcholine	$6·90 \times 10^{-4}$
2-Iodobenzoylcholine	$2·00 \times 10^{-4}$
2-Nitrobenzoylcholine	$3·38 \times 10^{-4}$
2-Methoxybenzoylcholine	$4·37 \times 10^{-4}$
2,6-Dichlorobenzoylcholine..	$4·63 \times 10^{-4}$
2,4,6-Tribromobenzoylcholine	$5·00 \times 10^{-4}$
2,4,6-Trinitrobenzoylcholine	$3·53 \times 10^{-4}$

(*a*) All compounds were used as the iodide salts.

Inhibition of acetylcholinesterase. Benzoylcholine is an inhibitor of acetycholinesterase (Glick, 1938). To obtain information about the effect of *ortho* substitution on the formation of an inhibitor enzyme complex the anti-acetylcholinesterase activity of the *ortho* substituted benzoylcholine compounds was determined. The standard Warburg

manometric technique was used, and the source of the enzyme was red cell stromata. The results obtained and the conditions used are given in Table IV.

DISCUSSION

Hydroxide Ion Catalysed Hydrolysis

The esters possessing one strongly electronegative chloro or nitro group in the *ortho* position were more rapidly hydrolysed than benzoylcholine. These results can be attributed to the electromeric properties of the substituents. There is a diminution of hydrolysis rates in the order chloro-, bromo-, and iodo-benzoylcholine. This is probably due to the smaller electromeric effects of the bromo and iodo substituents and also to steric effects (direct interactions) being involved with these larger groups.

The hydrolysis rates of *o*-methyl- and *o*-methoxy-benzoylcholine were both less than that of benzoylcholine. Both these substituents are able to force electrons into the carboxyl group thereby making hydroxide ion

TABLE V

RELATIVE RATES OF HYDROLYSIS OF *ortho* SUBSTITUTED BENZOYLCHOLINE COMPOUNDS AND *ortho* SUBSTITUTED ETHYL BENZOATE ESTERS

Benzoylcholine series		Ethyl benzoate series (Ingold, 1953c)	
Ortho substituent	Relative rate	*Ortho* substituent	Relative rate
H	1·00	H	1·00
Cl	1·40	Cl	2·20
NO_2	1·40	NO_2	8·70
Me	0·65	Me	0·125

attack difficult. The methyl group forces electrons towards the carboxyl group by induction because of its electropositive character, but the methoxy group does so by means of mesomerism.

Diortho substitution with chloro, bromo and methyl groups conferred great stability to hydrolysis. This almost certainly arises from the dominance of steric effects over induction and mesomerism. The instability of the dihydroxy ester is probably to be attributed to hydrogen bonding occurring between the hydroxyl groups and the carbonyl oxygen. This would increase the electropositivity of the carbonyl carbon atom. The hydrolysis of the trinitro ester is difficult to explain.

The relative rates of hydrolysis of several *ortho* substituted benzoylcholine compounds and the corresponding *ortho* substituted ethyl benzoates are compared in Table V. Although the conditions under which the hydrolysis rates were measured for each series were not identical there are noticeable differences in the relative values especially for the nitro and methyl substituted esters. The increased rate of hydrolysis of the methyl substituted benzoylcholine compared with the corresponding ethyl benzoate may arise from the attraction of electrons from the carbonyl carbon atom by the quaternary nitrogen atom. This effect has been demonstrated with aliphatic quaternary ammonium esters (Aksnes and

Prue, 1959). It was expected that the influence of the quaternary nitrogen atom would also cause an increase in the rate of hydrolysis of benzoylcholine esters possessing electronegative substitutents but this was not found. The quaternary nitrogen atom must again be involved since this is the only appreciable structural difference between the two series. In the case of *o*-nitrobenzoylcholine it is possible that the positively charged trimethylammonium group becomes associated with the negatively charged nitro group forming a loose ring configuration. In such a system the carbonyl carbon atom is shielded from attack by an hydroxide ion and hence the rate of hydrolysis of *o*-nitrobenzoylcholine would be slow compared with ethyl *o*-nitrobenzoate. A similar effect may also be expected with *o*-chlorobenzoylcholine but since chlorine is far less electronegative than the nitro group the effects would be expected to be much smaller.

Cholinesterase Catalysed Hydrolysis

Since all the mono substituted esters were hydrolysed it may be concluded that enzyme substrate complexes were formed. The ratios of the rates of cholinesterase catalysed hydrolysis, for the mono substituted esters, to those of non-enzymic hydrolysis are given in Table III. The ratio is small for those compounds with large *ortho* substituents. Hence it appears that cholinesterase catalysed hydrolysis is more susceptible to steric factors than is hydroxide ion catalysed hydrolysis.

Anti-acetylcholinesterase Activity

All the compounds prepared were more potent inhibitors of acetylcholinesterase than was benzoylcholine itself. This indicates that these esters form enzyme inhibitor complexes with acetylcholinesterase. It can be seen from Table IV that the inhibition of acetylcholinesterase is virtually independent of the size of the *ortho* substituent.

Conclusions

It is possible to prepare *ortho* substituted benzoylcholine compounds which form enzyme ester complexes with cholinesterase and with acetylcholinesterase but which are stable to cholinesterase catalysed hydrolysis. The groups which appear most suitable for ester stabilisation, when used as *ortho* substituents, are alkyl and iodo as mono substituents, and most groups, except hydroxy, as di-*ortho* substituents.

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THE ANTIPEPTIC ACTIVITY OF SULPHATED POLYSACCHARIDES

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Sulphated polysaccharides reduce the proteolytic activity of pepsin principally by reacting with the substrate rather than with the enzyme; and the complex so formed is protected by the polysaccharide from digestion.

THE claim by Babkin and Komarov (1932) that the "mucoitin- and chondroitin-sulphuric acid" components of gastric mucus are capable of diminishing peptic activity without altering the level of free acidity, and the subsequent interest in pepsin as a possible ulcerogenic factor received support from results (Matzner and Windwer, 1937; Schiffrin, 1940; Schiffrin and Warren, 1942) indicating that hydrochloric acid alone was not ulcerogenic but that pepsin, in the presence of hydrochloric acid at pH 1·1–1·5 (Schiffrin and Warren, 1942) was so. In the guinea-pig treated with large doses of histamine, Watt (1959) showed that the primary aetiologic factor in the initiation of the acute ulcerative process is the acid gastric juice. He claimed that the acidity was not as important as the excessive production and prolonged action of the acid gastric juice.

Antacid substances will inactivate pepsin by raising the pH and Shoch and Fogelson (1942) claimed that with the substances tested this was related to their influence on the acidity of the digest. Sodium lauryl sulphate, on the other hand, inhibited pepsin at high dilution without change in pH and increased the survival time of dogs given histamine.

The anionic properties of sodium lauryl sulphate are similar to those of sulphated polysaccharides and both will engage in salt formation with protein at or below the isoelectric point of the protein. Levey and Sheinfeld (1954) studied the inhibition of proteolytic action of pepsin by some sulphated polysaccharides, finding that *in vivo* and *in vitro* inhibition of peptic digestion occurred and they proceeded to show that oral chondroitin sulphate reduced the incidence of gastric ulcers in the Shay rat. These authors believed their results to indicate that the sulphated polysaccharide combined with the enzyme to form an inactive complex or one with reduced activity.

Tests by us with different sulphated polysaccharides did not support this interpretation and the antipeptic properties of some have been examined with the object of elucidating the way in which the peptic activity is reduced. The sulphated polysaccharide used in the present study has previously been reported to inhibit peptic activity and to prevent experimentally produced ulceration in the guinea-pig (Anderson and Watt, 1959a,b). Its antipeptic activity has been confirmed by Bonfils, Dubrasquet and Lambling (1959, 1960).

MATERIALS AND METHODS

Degraded carrageenan was used as a model. Similar experiments have also been done with heparin, fucoidan, chondroitin sulphate, laminaran sulphate and dextran sulphate.

*Degraded carrageenan.** The physical properties of undegraded carrageenan (Stoloff, 1959) (carrageenan of commerce) were not ideal for this study for several reasons. Carrageenan was degraded by careful treatment with dilute hydrochloric acid and subsequent precipitation after neutralisation with sodium hydroxide (British Patent 840,623). The product was an off-white powder containing 28–30 per cent of unchanged bound sulphate. Typical data are: S, 9·4 per cent; Ca, 0·2 per cent; Na, 7 per cent; K, 1·3 per cent; $[\alpha]_D + 34°$. The specific viscosities at 25° (M3 U-tube viscometer) of a 0·05 per cent aqueous solution of the parent carrageenan and the degraded carrageenan are 1·52 and 0·06 respectively. Degraded carrageenan is therefore considerably less viscous than carrageenan, and is readily and completely soluble in water and acid.

Pepsin. Crystallised pepsin, Armour Laboratories Ltd. *Toluidine blue.* Michrome brand (Edward Gurr Ltd.). *Gastric mucin.* Armour Laboratories Ltd. *Artificial gastric juice.* Solution of pepsin in hydrochloric acid. *Human gastric juice.* Samples from any one peptic ulcer patient pooled and centrifuged. The clear supernatant was used.

Measurement of Peptic Activity

Method A. Modified Anson (1938) *method.* Two flasks each containing 1 ml. of pepsin solution at pH 1·6 and 1 ml. solution of hydrochloric acid also at pH 1·6 were incubated at 35° for 10 min. and 1 ml. haemoglobin (Armour, for proteolytic enzyme assay) solution at pH 1·6 and 35° was then added and incubated for 30 min. In a further two flasks treated similarly the 1 ml. of acid solution was replaced by 1 ml. of solution of the sulphated polysaccharide at pH 1·6. After incubation, 10 ml. of 10 per cent w/v trichloracetic acid was added, the flasks allowed to stand in the water bath for 15 min. and the contents then filtered (Whatman No. 1). Five ml. of filtrate was added to 10 ml. N NaOH followed by 3 ml of a 1 + 2 dilution with water of Folin and Ciocalteu's reagent. The optical density of the blue colour developing in 10 min. was measured in an EEL absorptiometer using 1 cm. cells and 607 filter. Blanks were also included to measure the contribution to the colour of the pepsin, haemoglobin, and pepsin + inhibitor. When human gastric juice was used in this method, 2 ml. of centrifuged supernatant of the juice was used to dissolve the degraded carrageenan and to this was added the 1 ml. of substrate solution.

Method B. (Hunt, 1948). When the peptic activity of artificial gastric juice was measured by this method there was no further dilution of the juice before incubation. This was to allow a greater range of activity especially in studies involving inhibition. When degraded carrageenan was added it was dissolved in the centrifuged juice.

* Ebimar (Evans Medical Ltd.).

Uniform agitation is required throughout the digestion period to effect and maintain dispersion of the coarse precipitate formed between inhibitor and substrate.

Antipeptic Activity

This term is calculated from $S-I/S \times 100$ where S is optical density obtained in the determination of peptic activity from the treated digest without inhibitor, corrected for blanks as necessary; I is the corrected optical density for the treated digest with inhibitor. Antipeptic activity is thus the amount of inhibition, per cent.

Estimation of Degraded Carrageenan using Toluidine Blue

Degraded carrageenan reacts with toluidine blue like heparin does and the method of MacIntosh (1941) for heparin proved suitable for its

TABLE I

ANTIPEPTIC ACTIVITY AT DIFFERENT ENZYME LEVELS AND DIFFERENT SUBSTRATE: INHIBITOR RATIOS. SUBSTRATE: HAEMOGLOBIN (Hb). INHIBITOR: DEGRADED CARRAGEENAN (DC). INHIBITOR MIXED WITH ENZYME BEFORE ADDITION TO SUBSTRATE

Substrate (Hb) and inhibitor (DC) in reaction mixture			Antipeptic activity		
	Amount in mg./6 ml.		Pepsin added in amounts of		
Ratio	Hb	DC	50µg.	75 µg.	10 µg.
1	50	50	86	83	84
	10	10	34	20	7
5	50	10	39	40	37
10	50	5	21	23	21
	100	10	27	26	23
20	50	2·5	10	11	12
	200	10	20	22	21
30	50	1·7	5	8	9
	300	10	21	28	21

estimation. Optical densities were measured on a Hilger Spekker absorptiometer using filter OY2.

In the determination of degraded carrageenan remaining after reaction with protein, solutions in 0·2 per cent NaCl were made to contain 0·01 per cent w/v of degraded carrageenan and protein in amounts ranging from 0·005 per cent to 0·05 per cent w/v. The use of 0·2 per cent NaCl gave the same results as did 0·01 N HCl. The degraded carrageenan content was then estimated by calculating the toluidine blue removed from solution; this was assumed to be equivalent to the "free degraded carrageenan" which remained. The proteins studied were haemoglobin, human plasma protein, pepsin, and gastric mucoprotein.

RESULTS

Comparison of Sulphated Polysaccharides

The sulphated polysaccharides were estimated to have the following order of antipeptic activity, heparin, degraded carrageenan, fucoidan, chondroitin sulphate. Sodium sulphate and various non-sulphated polysaccharides (hyaluronic acid, agar, starch, tragacanth) were without

activity. The order of antipeptic activity found in the sulphated polysaccharides parallels the ester sulphate content of these substances.

Degraded Carageenan

Factors affecting the inhibition of pepsin. The duration of exposure and pH. Increasing the time of contact between pepsin and sulphated polysaccharide to 1 hr. did not modify the inhibition of peptic activity.

TABLE II

ANTIPEPTIC ACTIVITY AT DIFFERENT ENZYME LEVELS AND DIFFERENT SUBSTRATE: INHIBITOR RATIOS. SUBSTRATE: HAEMOGLOBIN (Hb). INHIBITOR: DEGRADED CARRAGEENAN (DC). INHIBITOR MIXED WITH SUBSTRATE BEFORE ADDITION OF ENZYME

Substrate (Hb) and inhibitor (DC) in reaction action			Antipeptic activity		
Ratio	Amount in mg./6 ml.		Pepsin added in amounts of		
	Hb	DC	50 µg.	75 µg.	100 µg.
1	50	50	91	87	84
	10	10	39	45	26
5	50	10	41	44	46
10	50	5	26	25	25
	100	10	14	19	21
20	50	2·5	15	12	14
	200	10	0	2	1
30	50	1·7	9	8	11
	300	10	3	8	16

TABLE III

ANTIPEPTIC ACTIVITY AT DIFFERENT ENZYME LEVELS AND DIFFERENT SUBSTRATE: INHIBITOR RATIOS. SUBSTRATE: PLASMA PROTEIN (PP). INHIBITOR: DEGRADED CARRAGEENAN (DC). INHIBITOR MIXED WITH ENZYME BEFORE ADDITION TO SUBSTRATE

Substrate (PP) and inhibitor (DC) in reaction mixture			Antipeptic activity		
Ratio	Amount in mg./6 ml.		Pepsin added in amounts of		
	PP	DC	100 µg.	200 µg.	300 µg.
1	50	50	82	85	86
	10	10	43	64	64
5	50	10	30	59	64
10	50	5	25	32	34
	100	10	26	29	29
20	50	2·5	15	21	19
	200	10	18	28	8
30	50	1·7	7	12	15
	300	10	9	14	11

Similarly, varying the pH of the medium between the limits pH 1·1-5·0 did not affect the inhibition.

The concentration of sulphated polysaccharide. Increasing the concentration of degraded carrageenan with either haemoglobin or plasma protein as substrate and constant enzyme and substrate levels caused an increasing inhibition of enzyme (Tables I, II, III and IV). The effect is independent of the enzyme concentrations employed; whether the inhibitor is mixed with substrate first or with enzyme first makes little or no difference. Peptic activity was measured using Method B.

The ratio of substrate (protein) to inhibitor (degraded carageenan). At different substrate concentrations similar variations in enzyme concentration did not alter the inhibition. Tables I to IV.

Experiments with gastric juice from ulcer patients. Similar experiments were made with human gastric juice, and the results are given in Tables V to VII.

Variation of the substrate (protein): inhibitor (degraded carageenan) ratio. Method A was used and it is seen in Table V that the antipeptic activity increases as the protein: degraded carrageenan ratio decreases.

Variation in enzyme concentration. Method A was used and Table VI shows that dilution of enzyme had little or no effect on antipeptic activity for a constant protein: degraded carrageenan ratio.

TABLE IV

ANTIPEPTIC ACTIVITY AT DIFFERENT ENZYME LEVELS AND DIFFERENT SUBSTRATE: INHIBITOR RATIOS. SUBSTRATE: PLASMA PROTEIN (PP). INHIBITOR: DEGRADED CARRAGEENAN (DC). INHIBITOR MIXED WITH SUBSTRATE BEFORE ADDITION OF ENZYME

Substrate (PP) and inhibitor (DC) in reaction mixture			Antipeptic activity		
Ratio	Amount in mg./6 ml.		Pepsin added in amounts of		
	PP	DC	100 μg.	200 μg.	300 μg.
1	50	50	88	89	88
	10	10	83	73	76
5	50	10	64	67	65
10	50	5	27	25	26
	100	10	35	31	34
20	50	2·5	13	14	13
	200	10	27	21	17
30	50	1·7	7	10	11
	300	10	24	16	10

TABLE V

VARIATION IN ANTIPEPTIC ACTIVITY OF DEGRADED CARRAGEENAN IN HUMAN GASTRIC JUICE WITH VARYING SUBSTRATE (PLASMA PROTEIN, PP): INHIBITOR (DEGRADED CARRAGEENAN, DC) RATIOS

Reaction mixture	Antipeptic activity	Ratio substrate: inhibitor
2 ml. gastric juice + 15 mg. DC + 120 mg. PP ..	36	8
2 ml. gastric juice + 15 mg. DC + 50 mg. PP ..	70	3·3
2 ml. gastric juice + 30 mg. DC + 50 mg. PP ..	85	1·7
2 ml. gastric juice + 30 mg. DC + 20 mg. PP ..	84	0·7

Variation of the substrate concentration. Method B was used with a bulked sample of gastric juice from patients with duodenal ulcer. The results are given in Table VII.

Reaction of Degraded Carrageenan with Proteins

A reaction between degraded carrageenan and proteins can readily be demonstrated by mixing acid solutions of both in suitable proportions when a precipitate may form which contains both substances. When the conditions of concentration or solubility do not allow the formation of a precipitate a decrease in the concentration of the free degraded carrageenan in solution can usually be detected. To demonstrate the relative affinity of degraded carrageenan for substrates and pepsin, under conditions where enzymic digestion is not employed, mixture of degraded

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carrageenan and each protein were prepared and the degraded carrageenan remaining in solution was estimated by the toluidine blue method. The relative affinities of a series of proteins for degraded carrageenan is shown in Fig. 1, from which it appears that the greater the protein: degraded carrageenan ratio the less the degraded carrageenan which is then available to react with the toluidine blue, giving a lower curve. When pepsin is the protein, a ratio of 5 causes the "removal" of less degraded carrageenan than when either haemoglobin or plasma protein

TABLE VI

Absence of effect on antipeptic activity, in human gastric juice, of variation in enzyme concentration for constant values of substrate (plasma protein, PP) and inhibitor (degraded carrageenan, DC)

Reaction mixture 3 ml.	Antipeptic activity	Ratio substrate: inhibition
2 ml. gastric juice + 20 mg. DC + 75 mg. PP ..	56	3·8
2 ml. gastric juice diluted 1 : 5 + 20 mg. DC + 75 mg. PP	62	3·8
2 ml. gastric juice diluted 1 : 10 + 20 mg. DC + 75 mg. PP	62	3·8

TABLE VII

Variation in antipeptic activity in human gastric juice with variation in substrate (plasma protein) concentration; i.e., alteration in substrate: inhibitor ratio

Degraded carrageenan mg./ml. juice	Antipeptic activity		
	mg. plasma protein substrate		
	140	280	420
35	93	75	84
30	89	69	70
25	84	69	63
20	77	57	57
10	51	43	18

is used in a ratio of 2. The reaction of degraded carrageenan with pepsin is small, less than with mucoprotein and much less than with the plasma protein or haemoglobin.

Discussion

Undegraded carrageenan of commerce yields viscous solutions at low concentrations, and hence might not be expected to disperse readily or react rapidly in the stomach, in the contents, or on the mucosa. For this reason carrageenan was degraded to give a product having the original ester sulphate content. It was only slightly less active in antipeptic property than purified heparin and has the significant advantage of being without anticoagulant effect.

Levey and Sheinfeld (1954) consider that the antipeptic properties of sulphated polysaccharides are the result of the action of these substances upon the protein enzyme. But if this were correct, it might reasonably be expected that inhibition of peptic activity could be effected with a

suitable sulphated polysaccharide in much lower concentration than is actually required, and the addition of excess quantities of substrate should not lower the amount of inhibition. On the contrary, it is clear from Tables I to IV that increasing the concentration of the substrate diminishes the inhibition and decreasing the enzyme concentration does not affect the amount of inhibition, which might be expected to have been increased had the reaction mainly been inactivating salt formation

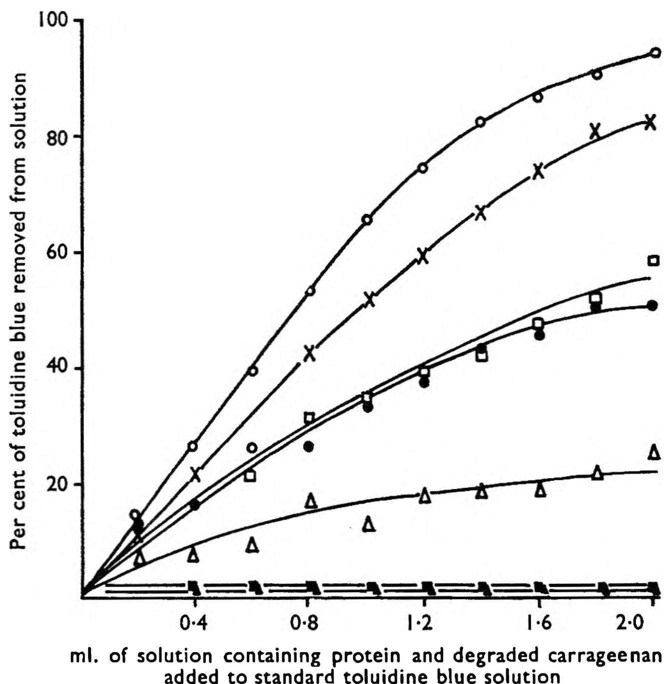

FIG. 1. The relative affinities of some proteins for degraded carrageenan.

		protein : degraded carrageenan
○——○	degraded carrageenan only	0
X——X	,, ,, + pepsin	5
□——□	,, ,, + plasma protein . .	2
●——●	,, ,, + mucoprotein . .	5
△——△	,, ,, + haemoglobin . .	2
■——■	,, ,, + plasma protein . .	5
▲——▲	,, ,, + haemoglobin . .	5

between enzyme and inhibitor. The ratio of protein substrate to sulphated polysaccharide appears to determine the inhibition. Furthermore, for similar ratios the total concentrations of each are shown to affect the inhibition, and this is probably so because of the nature of the complex salt formed. Important among these considerations would be its dissociation and solubility, as well as purely physical factors such as the ease or difficulty of mixing and diffusion of enzyme and soluble substrate in a medium in which floccules of varying size and amount are being formed.

These considerations are important because of the conditions frequently prevailing in the stomach which favour reaction of a sulphated polysaccharide with any protein present. Plasma protein and haemoglobin were thought to be realistic substrates because, apart from food protein, these two are likely to be encountered in the stomach or duodenum of the peptic ulcer patient. The reaction between sulphated polysaccharides involves salt formation (Jaques, 1943) and is instantaneous, and it might therefore be expected that upon presentation to an ulcerated surface, an immediate reaction would occur with deposition of the polysaccharide-protein complex and subsequent protection of the site from peptic erosion. On the basis of the results in Tables I to IV it is likely that the greater the dose of sulphated polysaccharide the greater the protection from peptic erosion, assuming the amount of protein remains constant. It therefore follows that if maximum deposition of the sulphated polysaccharide is to be obtained upon the sites to be protected the material should be administered at times when the food protein in the stomach is as low as possible.

From Fig. 1 it seems that the affinity for pepsin protein is lower than for plasma protein or haemoglobin or for gastric mucoprotein. This supports the conclusions of the digestion studies which indicate that the amount of pepsin present is not as important as the amount of substrate protein in determining the inhibition to be observed.

Increased pepsin concentrations occasionally gave slightly lower inhibition but this was never a consistent finding and the impression fitted with the results of the non-digestion study of the reaction between proteins and degraded carrageenan, which showed that some pepsin did react but that it was not a strong reaction. A precipitate of the pepsin-carrageenan complex was not obtained at the concentrations used in the digestion studies, although this in itself is no indication of absence of complex formation. The occasional lower inhibition at higher pepsin levels may reflect merely the greater chance of effective union of some enzyme with some substrate during the reaction between sulphated polysaccharide and substrate. Nevertheless, it appears that the inhibition of peptic activity is mediated by protection of the substrate, and it is probably desirable that this mode of action operates, because if pepsin is a causal factor in peptic ulcer the "substrate" becomes gastric or duodenal tissue. If now these tissue substrates are protected by an antipeptic substance complexed with mucoprotein the concentration of pepsin in the gastric juice and the loss of "free" sulphated polysaccharide due to gastric emptying become of small importance.

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THE DETERMINATION OF OESTRADIOL DIPROPIONATE IN OILS

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A method of assay of oestradiol dipropionate in pharmaceutical preparations is described in which interfering substances are removed from the solvent oils by partition chromatography. Oestradiol dipropionate is determined as oestradiol after acid hydrolysis of the oil solution using the iron phenol reagent.

Oestradiol dipropionate although not one of the most widely used oestrogens, nevertheless is manufactured and administered in large enough quantities to make a precise chemical determination a useful tool in both control and analytical work.

Difficulties have been experienced in the determination of oestradiol dipropionate in pharmaceutical preparations by the method described in U.S.P. XVI in that it has not been possible to duplicate results. The problem seems to lie in the final step of the method when an extraction of the coloured solution is made with ethyl ether. It appears that varying amounts of ether dissolve in the reagent causing dilution so that different values are obtained.

Because of this it appeared necessary to develop an alternate procedure. Since oestradiol dipropionate is administered parenterally as an oil solution the problem seemed to be to separate the oestrogen from the oil without carrying along substances which might subsequently interfere. The difficulty in this was to find a solvent partition system which would effectively separate the oestrogen from the oil.

A second approach was then tried in which the oestradiol dipropionate was hydrolysed and the free oestradiol was determined. This appeared to be the preferable procedure and was therefore used in the development of a method for this steroid.

Methods

Reagents

Chloroform—reagent grade; ethanol—absolute; isooctane—reagent grade: methanol—reagent grade; *n*-hexane—practical grade; light petroleum—ligroine, B.P. 30–60°, reagent grade; polyethyleneglycol 600—Carbowax 600, Carbide and Carbon Chemicals Co.; hydrochloric acid, concentrated—reagent grade; sulphuric acid—10 N; sulphuric acid—35 per cent; sodium hydroxide—N; Celite No. 545—Johns Manville; iron-phenol reagent—U.S.P. XVI, page 277.

Celite Column

The column used to remove interfering substances from the oil solvent is a modification of one used by Theivagt and Campbell (1959) to separate vitamin D from vitamin A in multivitamin mixtures.

Five g. of Celite No. 545 is wetted with 25 ml. of isooctane and 2 ml. of polyethyleneglycol 600. The whole is vigorously stirred until the mixture is thick in consistency. It is then transferred to a column, 10 mm. in diameter, fitted with a coarse porosity fritted disc at one end, and firmly packed down. The column is then ready for use.

Preparation of Extract

A sample of oestradiol dipropionate in oil equivalent to 1·0 mg. of the steroid (1–2 ml.) is diluted with 5 ml. of *n*-hexane and quantitatively

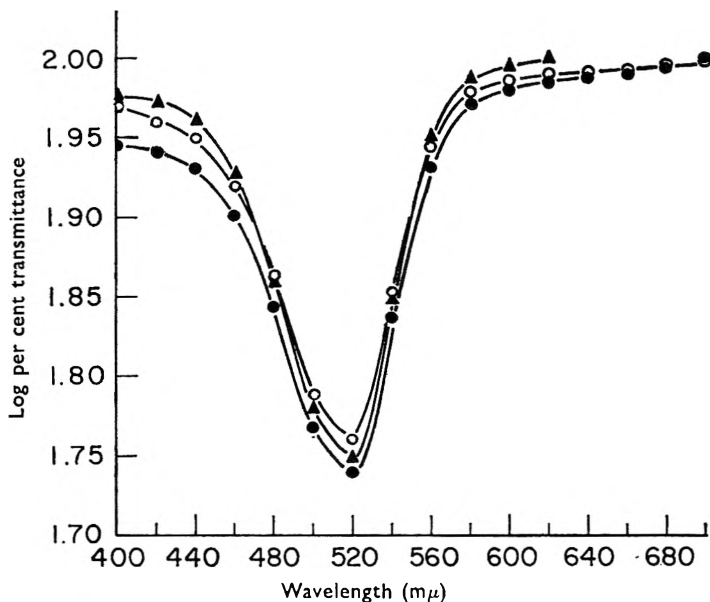

FIG. 1. Absorption curves for extract of hydrolysate of oestradiol dipropionate in sesame oil before and after column treatment.
▲ 15 mg. oestradiol.
● Oestradiol dipropionate in sesame oil 14·16 mg. oestradiol eq. before column.
○ Oestradiol dipropionate in sesame oil 14·16 mg. oestradiol eq. after column.

transferred to a celite column prepared as described. The oily solution is washed into and through the column with small portions of *n*-hexane until a total of 75 ml. has been used. The eluate is collected 25 ml. at a time and evaporated in a 50 ml. round bottomed flask with a standard taper ground joint on the neck. The *n*-hexane is evaporated from the oil on a steam bath under a current of air. Ten ml. of methanol, 0·5 ml. of concentrated hydrochloric acid and two glass beads are added to the flask and the mixture is refluxed on a steam bath for 15 min. The condenser is removed and while the flask is still on the steam bath the methanol and a large part of the acid is driven off under a stream of air. When the oily residue stops bubbling and looks dry it is transferred to a 125 ml. separatory funnel with four 15 ml. portions of light petroleum.

The light petroleum solution is extracted five times with 10 ml. portions of N sodium hydroxide. The flask which contained the hydrolysate is rinsed with the portions of sodium hydroxide before adding to the separatory funnel. The combined alkaline extracts are acidified carefully with 10N sulphuric acid to pH 2–3 and allowed to stand for 1hr. After

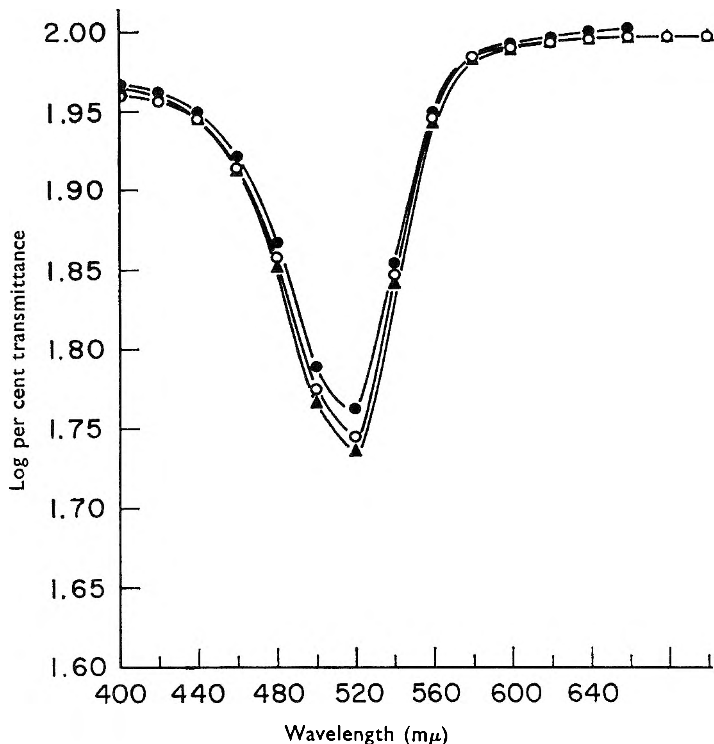

FIG. 2. Absorption curve for extract of hydrolysate of oestradiol dipropionate in corn oil before and after column treatment.

▲ 15 mg. oestradiol.
● Oestradiol dipropionate in corn oil to 14·16 mg. oestradiol eq. before column.
○ Oestradiol dipropionate in corn oil to 14·16 m.g. oestradiol eq. after column.

checking to make sure the mixture is still acid it is extracted with four 15 ml. portions of chloroform. The chloroform extracts are run into a 100 ml. volumetric flask and taken to dryness on a steam bath. The residue is then dissolved in ethanol and the flask is made up to volume with the same solvent. Aliquots of this solution are taken for the determination of oestradiol.

Determination of Oestradiol

Oestradiol is determined by the U.S.P. XIV method, parts of which have been modified to suit conditions in this laboratory.

An aliquot of the extract (prepared above) equivalent to 10–20 μg.

oestradiol dipropionate is transferred to a 10 ml. volumetric flask and taken to dryness on a boiling water bath. Aliquots of a standard solution of oestradiol also in ethanol are taken so that 5, 10, 15 and 20 μg. of the steroid are placed in four separate flasks. These aliquots are also taken to dryness.

A blank is prepared by evaporating 1 ml. of ethanol in a 10 ml. volumetric flask and then carrying it through the same procedure as for the sample or standard.

To each flask is added 1·5 ml. of the iron-phenol reagent The flasks are allowed to stand for 30 min. with frequent shaking and are then placed

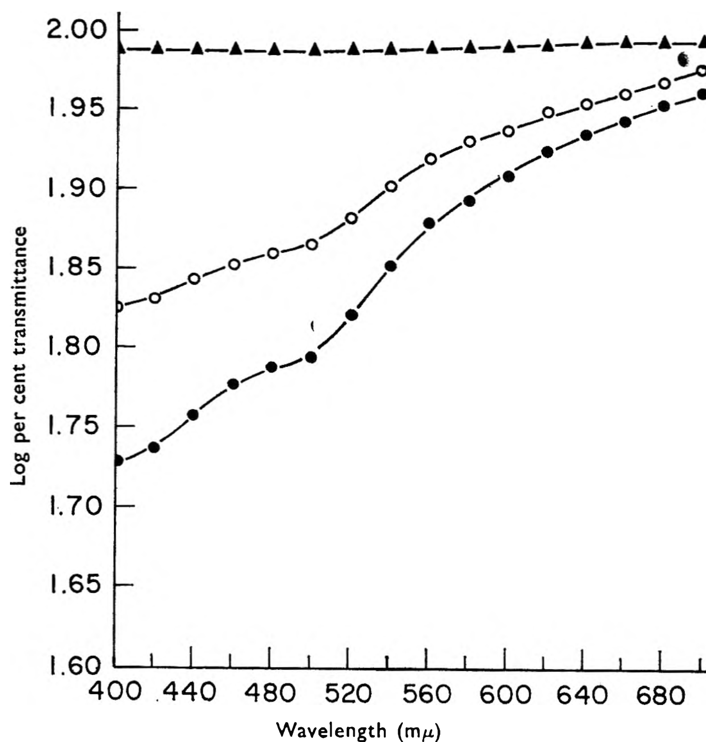

FIG. 3. Absorption curves for extract of hydrolysate of sesame oil before and after column treatment.

○ 1 ml. sesame oil before column treatment.
● 2 ml. sesame oil before column treatment.
▲ 1 ml. seame oil after column treatment.

in a boiling bath. After heating for 2–3 min. they are stoppered and after 5 min. are again well shaken. The heating is continued for 1 hr. At the end of this time the flasks are thoroughly cooled and 6 ml. of 35 per cent sulphuric acid is added to each. They are well shaken and after transferring to appropriate cuvettes are read against the blank in a Coleman Junior spectrophotometer at 520 mμ.

The amount of oestradiol in the aliquot of the extract is found by comparison with the standard curve. It is then calculated in terms of ostradiol dipropionate by multiplying by a factor of 1·4117.

Results and Discussion

Hydrolysis

In preliminary work it was found that alkaline hydrolysis caused saponification of the oil and troublesome emulsions occurred during the subsequent separation of oestradiol from the hydrolysate. With a change to acid hydrolysis employing hydrochloric acid this difficulty was overcome and the oestradiol was easily separated. However, when the amount of oestradiol present in hydrolysates of standard solutions was determined, variable recoveries in terms of oestradiol dipropionate were found. These recoveries were higher than 100 per cent when sesame oil was used and usually lower with corn, cottonseed and peanut oils.

TABLE I

Recovery of oestradiol dipropionate from oil solutions before and after column treatment

Oil	Typical recovery per cent	
	Before column	After column
Sesame	115·1	102·0
	113·5	99·6
Corn	93·7	100·7
	94·3	101·3
Cottonseed	73·3	99·0
	97·0	100·6
Peanut	97·2	100·8
	99·0	102·4

Removal of Interfering Substances

With an extract of the hydrolysate of oestradiol dipropionate in sesame oil an interfering colour developed which gave an absorption curve slightly different than that for standard oestradiol. However, if the sesame oil sample containing the oestradiol dipropionate is first put through the Celite column described above this interfering colour does not develop. The removal of this interference can be clearly seen in Fig. 1. This interference has previously been noted by Dracass and Foster (1943) when determining stilboestrol dipropionate in oily solution.

With corn, cottonseed and peanut oils a lower recovery than 100 per cent is generally found with an absorption curve that is identical in all respects with that of oestradiol. The curves shown for corn oil in Fig. 2. both before and after column treatment are typical of cottonseed and peanut oils also.

Apparently in these instances there is something in the oil which inhibits full colour development of the oestradiol with the iron-phenol reagent. When these oil solutions are put through the same column as that used with sesame oil the inhibitors are removed and it can be seen in Table I that a good recovery of oestradiol dipropionate is achieved.

Typical recoveries of oestradiol dipropionate from commercial preparations are shown in Table II.

When aliquots of 1 and 2 ml. of sesame oil alone were put through the same procedure as for oestradiol dipropionate in oil there was definite colour formation with the iron-phenol reagent. If, however, the samples of oil were first put through the column it is clearly shown in Fig. 3 that the substances causing interfering colour formation were removed.

TABLE II

RECOVERY OF OESTRADIOL DIPROPIONATE IN MULTIPLE ASSAYS OF COMMERCIAL PREPARATIONS

Preparation	Labelled	Found
A	1 mg./ml.	0·97 mg./ml.
		0·99 ,,
		0·98 ,,
		0·98 ,,
B	1 mg./ml.	1·06 ,,
		1·04 ,,
		1·00 ,,
		1·02 ,,

When the same procedure was carried out with corn, cottonseed and peanut oils there was no interfering colour formation either before or after column treatment.

These interfering substances are not artifacts produced by the acid hydrolysis of an oil solution since they are present to the same extent in aliquots of oil which have been extracted without being put through the hydrolysis procedure. Neither are they oxidation products from oil constituents since rancid oils do not contain any more of these interfering components than fresh oils.

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THE ANATOMY OF THE LEAF OF *SYMPHYTUM OFFICINALE* L.

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The history and uses of the leaves of *Symphytum officinale* L. are given together with an illustrated account of the macroscopy of the upper and lower leaves of the plant and of the anatomical structure of the leaf. The diagnostic characters of the powdered leaves are also recorded and illustrated.

COMFREY has been used in medicine for many centuries. It was used for healing broken tissue and bone, hence its Greek name Συμφυΐον derived from δυμουω, to unite (Barton and Castle 1877). The brief morphology and medicinal uses of two species of *Symphytum* (*S. petraeum* and *S. alterum*) are described in Dioscorides' Herbal (*ca.* A.D. 100) (Gunther, 1959). Parkinson (1640) stated that the plant is *S. petraeum*, whereas Turner (1548) and Matthioli (1598) used the specific name *S. alterum*. The modern name, Comfrey, derives from the mediaeval Latin *Comfiria*, which in turn replaced the Latin *Conferva* of Pliny (Grigson, 1955). It was also known as *Consolida maior* and, by the Romans, *Solidago* (Dodoens, 1586; Salmon, 1610). The Romans took the plant to northern Europe (Kamm, 1938), and it was used by the Saxons as a vulnerary (Martindale, 1924). By A.D. 1000 it had appeared in the monastery lists and leech-books (Kamm, 1938). From the Middle Ages to the middle of the nineteenth century, there was a belief that both the roots and leaves had the power to heal wounds and bones (Dodoens, 1586, Salmon, 1610). This resulted in the use of names such as knit-back, bone set, nit-bone, healing blade, bruisewort (Kamm, 1938) and blackwort (Gerard, 1633). It was widely used for quinsy, whooping cough, in poultices for bruises and open wounds and also as a styptic or a pectoral (Kamm, 1938).

In 1912 it was found to be of value in the formation of epithelial tissue in external ulceration and in ulcers of the stomach and duodenum, due to the presence of allantoin (Bramwell, 1912; Wood and Lawall, 1926). The infusion is still used as a fomentation for reducing inflammations associated with sprains and bruises and as a medicine for chest complaints. It is widely used in country districts, particularly Warwickshire (Min. Agric. Fish., 1941), and is generally available commercially.

The British Pharmaceutical Codex of 1934 introduced a monograph for the leaf, but current and recent textbooks refer to the drug only as an adulterant of the leaf of *Digitalis purpurea* (Perrot, 1943; Trease 1957, Wallis, 1960). The only detailed work on the leaf is that of Bider (1935); this is not illustrated and deals only with macroscopical features together with histological epidermal features, particularly the trichomes. Kay (1938) has briefly described and illustrated the covering trichomes.

In view of the fact that no detailed anatomical work is evident, and the well-known use of the plant in medicine and agriculture, this communication describes the detailed structure of the leaf, to establish the diagnostic characters necessary for its identification in the whole or powdered condition.

Material

The material used for the work was collected from wild plants growing in areas near Bradford. Further material was obtained from plants by transplanting roots from Yorkshire and propagated vegetatively in Stapleford, Nottinghamshire. All plants used in the present work possessed the characters typical of the species.

Methods of Investigation

Chloral hydrate solution proved satisfactory for epidermal preparations; systematic serial sections were prepared by methods described previously (Fell and Rowson, 1955, 1956), employing polyethylene glycols and freezing microtomy.

Sections were mounted in chloral hydrate solution or dilute glycerol when making drawings of parenchymatous tissues, in phloroglucin and hydrochloric acid when investigating the nature of the vascular tissues, and chloral-iodine to demonstrate the presence of micro-starch. Ruthenium red and methylene blue solutions gave positive reactions with mucilage in the cells of the upper and lower epidermises of the interneural lamina and midrib, also the pericyclic collenchyma of the midrib; negative reactions were given by corallin soda solution and 0·02 N iodine. A negative result for tannins was obtained on the addition of 5 per cent ferric chloride solution to sections of the leaf.

An investigation was made into the nature of numerous crystals present in the bases of many trichomes, epidermal cells, phloem and, to some extent, in the remaining parenchymatous tissue. They did not dissolve in phloroglucin and concentrated hydrochloric acid, or on the addition of 66 per cent w/w sulphuric acid. They were found to be birefringent when examined in polarised light after mounting in dilute glycerol or chloral hydrate solutions, or even after the addition of strong acid. Since, therefore, the crystals were obviously not calcium carbonate or oxalate, it was thought that they may be silica or allantoin. Mothes and Engelbrecht (1954) have proved that allantoin occurred in the leaves. Vogl (1918) found monoclinic crystals of allantoin in the rhizome of *S. officinale*. He mounted sections of the rhizome in 20 per cent acetic acid in alcohol, covered, and sealed the glass with paraffin, when further recrystallisation was effected. This could not be demonstrated in the leaves examined; since these had been preserved in ethanol, it was assumed that all the allantoin present had already crystallised out.

Schlepegrall (1892) and Titherley and Coppin (1912) identified allantoin in the root of *S. officinale*. On the addition of mercuric nitrate solution, a white precipitate was produced; the same method was used with scarlet runner beans by Power and Salway (1913). When sections and surface

preparations were treated with this reagent, a white precipitate and occasionally a red colour was produced with some crystals, particularly those in the base of the trichomes, indicating that a proteinous substance was present.

Comfrey root contains a little tannin. An aqueous extract of the leaves was treated with 95 per cent ethanol saturated with sodium chloride, and the filtrate was tested with 5 per cent ferric chloride solution and 1 per cent iron and ammonium citrate solution, but no positive results

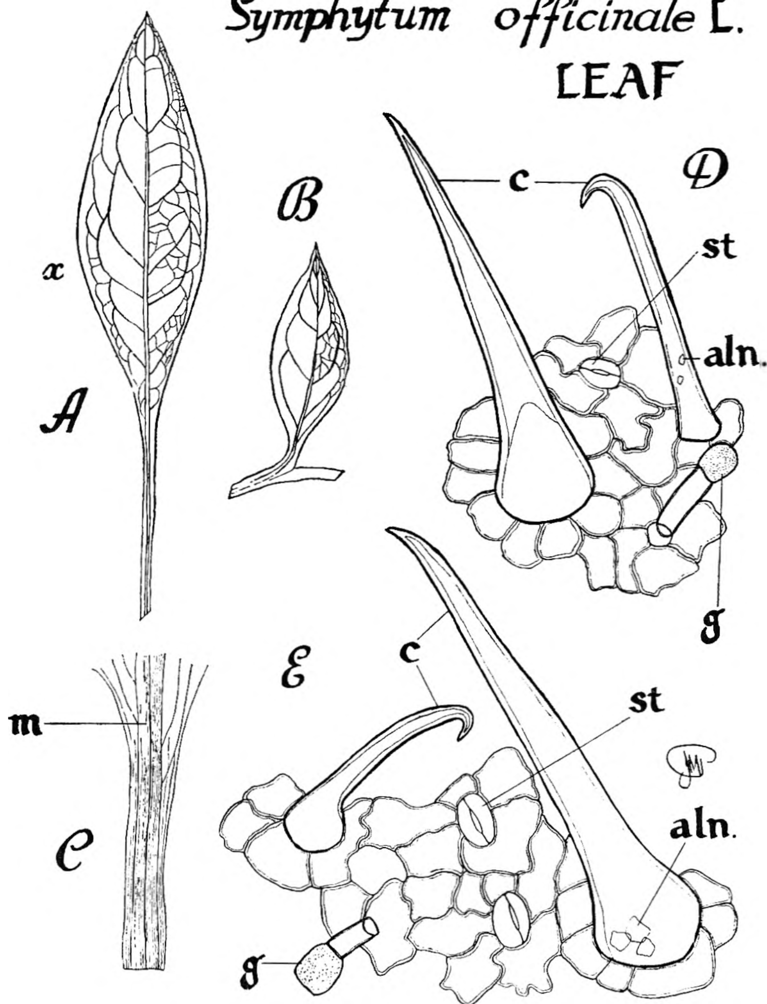

FIG. 1. Leaf of *Symphytum officinale* L. *A*, complete lower petiolate leaf. *B*, Complete upper sessile leaf. *C*, enlargement of petiole, showing decurrent lamina and lateral veins. *D*, upper epidermis of lamina. *E*, lower epidermis of lamina. $A \times \frac{1}{4}$; $B \times \frac{1}{4}$; $C \times 1$; *D* and $E \times 200$. aln., allantoin crystals; c, covering trichome; g, glandular trichome; m, midrib; st, stoma. *x*, position at which transverse section illustrated by Fig. 2, A, was made.

were obtained. The mucilage obtained by precipitation was washed with water to free it from the salt; treatment with ruthenium red and methylene blue gave positive results, whereas corallin soda and 0·02 N iodine gave negative reactions.

MACROSCOPY

Symphytum officinale L. is an erect hispid perennial, producing erect stems 2–3 ft. in height; both cauline and radical leaves are present. The plant grows in damp places especially near rivers and streams.

The leaf is simple, broadly lanceolate in shape, about 10–20 cm. in length and 3–4 cm. wide, with an acute apex and an entire or slightly wavy margin. The lateral veins anastomose towards the margin of the leaf; this feature shows more prominently on the under surface (Fig. 1, *A*).

The lower leaves are petiolate. The petiole is winged and the lateral veins of the leaf run parallel with the midrib, as far as the junction of the petiole with the stem (Fig. 1 *A* and *C*).

The upper leaves are sessile, the base being decurrent with the stem. They are similar in shape and features to the lower leaves, but are about half their size (Fig. 1, *B*).

The leaves have a brownish-green colour, no characteristic odour, a slightly astringent taste and the surface is hispid.

ANATOMICAL STRUCTURE

LEAVES

Lamina, interneural region (Fig. 1, *D* and *E*; Fig. 2, *B*)

The UPPER EPIDERMIS is covered with a thin smooth cuticle and consists of one layer of polygonal cells having sinuous anticlinal walls. They measure about *H 22 μ and Lev L and B 36 μ to 94 μ. *Stomata* occur fairly frequently and are of the anisocytic type. They are level with the epidermis, circular to oval in outline and have a prominent central pore. The circular stomata are about 25 μ in diameter and the oval ones about 25 μ to 36 μ long, 14 μ to 25 μ wide (Fig. 1, *D*; Fig. 2, *B*). Hydathodes are absent, mucilage is present in the cells, also numerous crystals of allantoin which are irregularly shaped but measure about 5 μ in any diameter (Fig. 2, *B*).

Covering trichomes are numerous, may be of two types and arise from both veins and interneural epidermises. The more common type has unicellular, straight, thick, cellulose walls, tapering to an acute apex. The lumen is visible throughout the entire length; the base is swollen and often contains several crystals of allantoin. The trichomes measure about 365 μ to 880 μ in length and 43 μ to 90 μ wide at the base, which

* The symbols H, Lev, Lev L and Lev B are suggested for the purpose of describing organs showing bilateral symmetry by Moll and Janssonius (1923a). The symbol H = height in a direction perpendicular to the surface of the organ; Lev = in the direction of the surface of the organ; Lev L and Lev B = parallel to the surface at the same time in a longitudinal or transverse direction respectively.

is surrounded by about 8 to 12 small epidermal cells (Fig. 1, *D*; Fig. 5). The second type of covering trichome which is characteristically hooked occurs infrequently and may even be absent. They are unicellular, straight except for the hooked acute apex and have thick cellulose walls. They measure about 115 μ to 240 μ in length and 29 μ to 36μ wide at

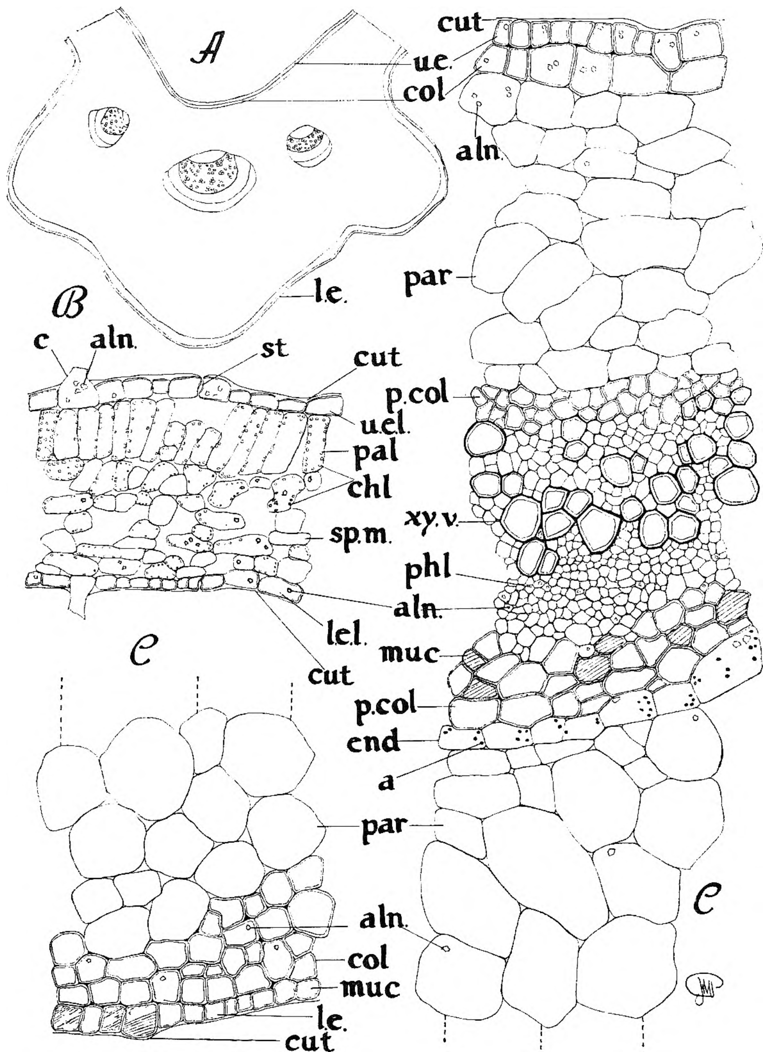

FIG. 2. Leaf of *Symphytum officinale* L. *A*, transverse section of midrib, cut at position *x* (see Fig. 1, *A*). *B*, transverse section of lamina, interneural region. *C*, central region of *A*. *A* × 25; *B* and *C* × 200. *a*, starch; aln., allantoin crystals; c, covering trichome; chl, chloroplast; col, collenchyma; cut, cuticle; end, endodermis; l.e., lower epidermis; l.e.l. lower epidermis lamina; muc, mucilage; pal, palisade; par, parenchyma; p.col, pericyclic collenchyma; phl, phloem; sp.m., spongy mesophyll; st, stoma; u.e., upper epidermis; u.e.l., upper epidermis of lamina; xy.v., xylem vessel.

the base; the base is swollen and may contain crystals of allantoin (Fig. 1, *D*).

Glandular trichomes are also present, but are less numerous than the covering type. They consist of a unicellular stalk, and a unicellular, sub-spherical glandular head. They are about 57 μ to 90 μ long and the head has a diameter of about 21 μ to 36 μ and occur on both veins and interneural epidermis (Fig. 1, *D*).

The MESOPHYLL is clearly differentiated. The *palisade* consists of a single layer of cells, which is not continuous across the midrib. The individual cells are cylindrical and measure about H 36 μ to 61 μ, Lev L and B 11 μ to 22 μ and contain chloroplasts (Fig. 2, *B*). Immediately below the palisade are four to six rows of *spongy mesophyll.* They are loosely arranged, are both rounded and elongated in surface view and measure about H 14 μ to 29 μ and Lev L and B 22 μ to 47 μ. They contain chloroplasts, and many crystals of allantoin (Fig. 2, *B*; Fig. 3, *A*).

The LOWER EPIDERMIS also has a thin smooth cuticle. Its cells measure about H 11 μ to 18 μ and Lev L and B 18 μ to 43 μ. The anticlinal walls are more strongly wavy than those of the cells of the upper epidermis. *Stomata* are very numerous, of the anisocytic type and oval in shape. They measure about 22 μ to 26 μ in length and 18 μ to 25 μ wide (Fig. 1, *E*). Mucilage and numerous crystals of allantoin are present in these cells (Fig. 2, *B*).

Covering trichomes are numerous and are similar to those present on the upper interneural epidermis. The straight conical covering trichomes on the lower surface are shorter than those of similar type on the upper surface. They measure about 190 μ to 560 μ in length and 36 μ to 90 μ wide at the base (Fig. 1, *E*). The hooked covering trichomes are similar in character to those on the upper interneural surface, but are more numerous (Fig. 1, *E*).

Glandular trichomes are rare, occasional ones are found on the veins; they have similar structures to those found on the upper interneural epidermis (Fig. 1, *E*).

Midrib (Fig. 2, *A* and *C*; Fig. 3, *B* to *F*)

The midrib has a typically dicotyledonous structure, the only variation being the number of individual bundles present in transverse sections from apex to the base of the leaf. Serial sections showed that one bundle occurs at the apex and about 12 at the base, this increase being due to the entrance of the successive lateral veins into the central area of the meristele.

The UPPER EPIDERMIS is covered by a thin cuticle and consists of a single layer of straight walled, elongated polygonal cells measuring about H 25 μ to 50 μ, Lev B 25 μ to 50 μ and Lev L 65 μ to 180 μ. Numerous crystals of allantoin are present in the cells, also mucilage (Fig. 2, *C*; Fig. 3, *B*).

Stomata, when present, measure about 28 μ to 33 μ in length and 25 μ to 28 μ wide, but were absent in about one-half of the specimens examined.

Covering trichomes are fairly frequent and similar in character to those of the upper interneural epidermis. Hooked trichomes are either absent or infrequent, and are similar to those on the upper interneural epidermis.

Glandular trichomes are numerous, measuring about 79 μ to 115 μ in length. They consist of a unicellular or occasionally bicellular stalk and a unicellular subspherical glandular head about 25 μ to 32 μ in diameter (Fig. 3, *B* and *D*).

The LOWER EPIDERMIS has a thin, smooth cuticle and consists of straight walled, elongated polygonal cells measuring about H 11 μ to 36 μ, Lev B 11 μ to 36 μ and Lev L 75 μ to 180 μ. Numerous crystals of allantoin and mucilage are present in the cells of this layer (Fig. 2, *C*; Fig. 3, *C*).

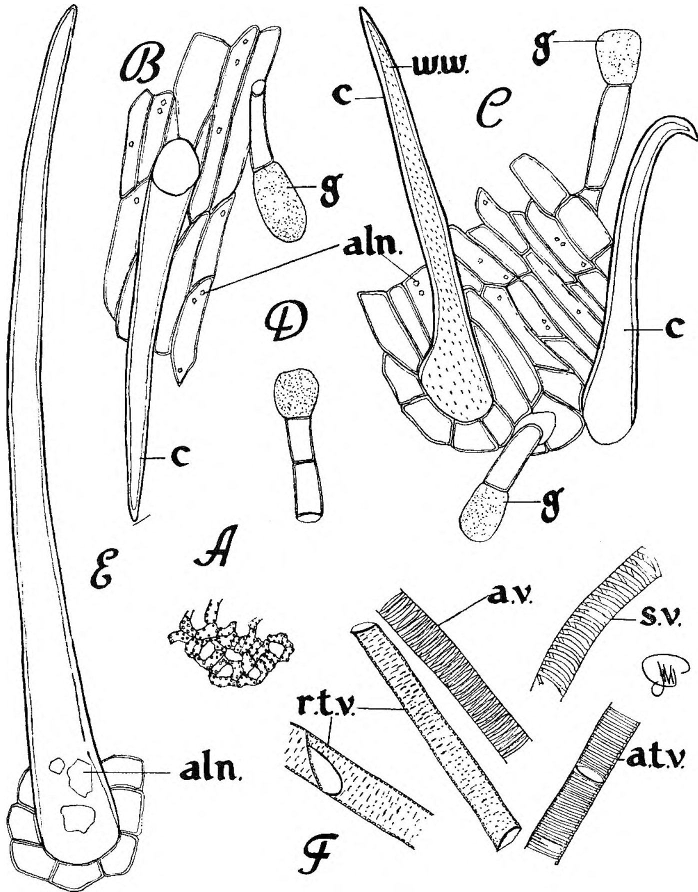

FIG. 3. Leaf of *Symphytum officinale* L. *A*, spongy mesophyll in surface view. *B*, upper epidermis of midrib. *C*, lower epidermis of midrib. *D*, glandular trichome with a bicellular stalk from upper epidermis of midrib. *E*, covering trichome from lower epidermis of midrib. *F*, isolated elements obtained by maceration of the midrib. *A*, *B*, *C*, *D* and *F* × 200; *E* × 100. aln., allantoin crystals; a.t.v., annular tracheidal vessel; a.v., annular vessel; c, covering trichome; g, glandular trichome; r.t.v., pitted tracheidal vessel; s.v., spiral vessel; w.w., warty wall.

Covering trichomes are frequent, similar in character to those of the lower interneural epidermis, but much larger. They measure about 468 μ to 1,140 μ in length and 54 μ to 108 μ wide at the base. Their walls are cellulosic in nature, staining blue with 0·02 N iodine followed by 66 per cent w/w sulphuric acid. Some trichomes have acute apices, others have blunt ones; the walls of some trichomes are warty. Crystals of allantoin may be present in the base of these trichomes (Fig. 3, *C* and *E*). Hooked covering trichomes occur in varying frequency; they are similar in structure to those on the upper epidermis of the interneural lamina (Fig. 3, *C*).

Glandular trichomes are frequent and are larger than those on the upper midrib epidermis. They consist of a bicellular stalk, the lower cell being much smaller than the upper, and a unicellular sub-spherical glandular head. They measure about 94 μ to 162 μ in length and the glandular head is about 25 μ to 36 μ in diameter (Fig. 3, *C*). Glandular trichomes of a similar type to those on the upper interneural epidermis are also present.

Stomata, similar in structure to those on the upper epidermis of the midrib, occur.

The CORTEX contains one or two rows of hypodermal *collenchyma* below the upper epidermis and one to three rows above the lower epidermis. Chloroplasts are absent, but occasional crystals of allantoin are present. The remainder of the cortex is parenchymatous (Fig. 2, *C*, Fig. 3).

An *endodermis* (or starch sheath) is present and is approximately horse-shoe shaped in transverse section. It consists of one layer of cells containing minute starch grains, immediately adjacent to the pericyclic region. No other layer contains starch grains (Fig. 2, *C*). Despite the fact that the layer does not completely surround the meristele, such an arrangement may be regarded as an endodermis (cf. the endodermis of Althaea (Moll and Janssonius, 1923b).

The MERISTELE is round to oval in shape and well defined in transverse section (Fig. 2, *A*).

The PERICYCLE consists of a well-defined area of collenchyma above the xylem and below the phloem. Most of the cells contain mucilage (Fig. 2, *C*).

The PHLOEM is composed of sieve tissue and parenchyma. The individual sieve-tube elements measure about 100 μ to 200 μ in length and about 10 μ in diameter, with transverse sieve plates. The companion cells are narrow, but quite well defined in longitudinal section. The medullary rays are not clearly defined. Numerous crystals of allantoin are present in this region (Fig. 2, *C*).

The XYLEM consists of vessels and tracheidal vessels occurring singly or in small groups. The vessels show annular and spiral thickening and measure about 15 μ to 30 μ in diameter. The tracheidal vessels have annular and pitted thickening. There is also a large amount of un-lignified parenchyma (Fig. 2, *C*; Fig. 3, *F*).

Lateral veins exhibit similar anatomy to that of the midrib.

PETIOLE

The petiole is about 6 to 10 cm. long and 4 to 6 mm. wide; the lamina is decurrent on both sides throughout its entire length (Fig. 1, *A* and *C*). A transverse section through the petiole shows an arc of up to 12 bundles, some of which have arisen by the anastomosis of two to four small bundles from the lateral veins in the leaf. These run parallel with the mid-rib in the central stele. Only two or three small veins run down either side of the decurrent lamina, again parallel with the midrib.

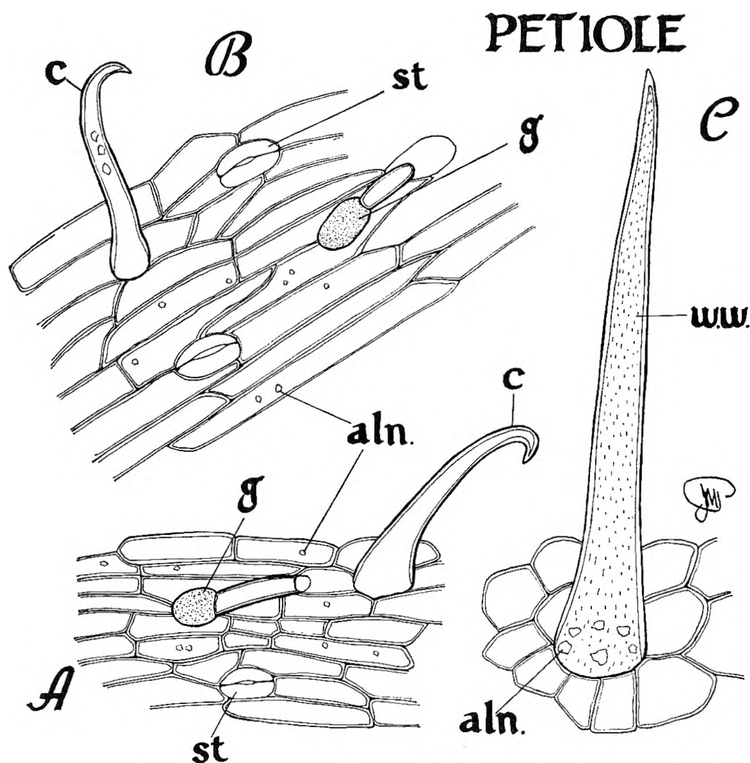

FIG. 4. Petiole of *Symphytum officinale* L. *A*, upper epidermis of petiole. *B*, lower epidermis of petiole. *C*, covering trichome from either lower or upper epidermis of petiole. *A* and *B* × 200. *C* × 100. aln., allantoin crystals; c, covering trichome; g, glandular trichome; st, stoma; w.w., warty wall.

The UPPER EPIDERMIS has cells possessing a similar structure to those of the mid-rib epidermis. They contain mucilage and crystals of allantoin (Fig. 4, *A*). *Stomata* normally occur quite frequently; they measure about 36 μ to 43 μ in length and are orientated in the direction of the long axis of the epidermal cells (Fig. 4, *A*). *Covering trichomes* are frequent and are of two types. Trichomes, similar to the straight covering ones present on the upper epidermis of the mid-rib, occur, but are much longer; they measure about 396 μ to 1,584 μ in length and

about 72 μ to 127 μ wide at the base, which is usually swollen and surrounded by 8 to 12 small epidermal cells. Some trichomes have warty walls (Fig. 4, *C*). The hooked covering trichomes may also be present but their occurrence is variable; they are similar in dimensions to those on the mid-rib epidermis (Fig. 4, *A*).

Glandular trichomes are frequent on this surface and measure about 90 μ to 137 μ in length. They consist of a unicellular or occasionally bicellular stalk and have a unicellular, subspherical glandular head measuring about 22 μ to 36 μ (Fig. 4, *A*).

The LOWER EPIDERMIS exhibits a thin, smooth cuticle, and is composed of polygonal, elongated straight-walled cells, measuring about H 18 μ to 33 μ, Lev B 14 μ to 33 μ and Lev L 97 μ to 396 μ; they contain mucilage and crystals of allantoin (Fig. 4, *B*). *Stomata* occur frequently and are orientated in the direction of the epidermal cells; they are oval in shape and measure about 36 μ to 47 μ in length and 22 μ to 29 μ in diameter (Fig. 4, *B*).

Covering trichomes are frequent and are of two types, both types being equally abundant. The straight type are similar to those present on the lower epidermis of the mid-rib, some trichomes have warty walls. They have thick cellulose walls and an acute apex, and measure about 162 μ to 1,620 μ in length and about 43 μ to 151 μ at the base, which is usually swollen and may contain crystals of allantoin (Fig. 4, *B*). The second type of covering trichome is hooked, similar to those present on the lower epidermis of the mid-rib, but are longer and measure about 162 μ to 378 μ in length and 25 μ to 58 μ wide at the base (Fig. 4, *B*).

A transverse section through the petiole is similar to that through the mid-rib.

The CORTEX is composed of *collenchyma* and *parenchyma*; below the upper epidermis are two or three layers and above the lower epidermis are three or four layers of cortical collenchyma. The remainder of the cortex is parenchymatous.

Each bundle has a horse-shoe shaped endodermis immediately adjacent to the lower pericyclic region. The cells are parenchymatous and contain minute starch grains.

The PERICYCLE consists of a well defined area of collenchyma below the phloem and above the xylem.

The PHLOEM is similar in structure to that of the mid-rib.

The XYLEM consists of single or small groups of vessels and tracheidal vessels; there are no distinct medullary rays. The vessels show annular and spiral thickening, but the tracheidal vessels are almost entirely reticulately thickened. There is a large amount of unlignified xylem parenchyma.

Calcium oxalate crystals are absent, and the presence of calcareous or silicaceous deposits could not be demonstrated in any trichome.

POWDER

A No. 44 powder has a green colour, no odour and only a slightly astringent taste.

The powder was examined using the following reagents: dilute glycerol, chloral hydrate solution, phloroglucin and concentrated hydrochloric acid, chloral iodine, ruthenium red and methylene blue. The diagnostic features (Fig. 5) are:—

Large numbers of *covering trichomes*, either in whole or broken condition, or still attached to pieces of epidermis. Fragments up to about

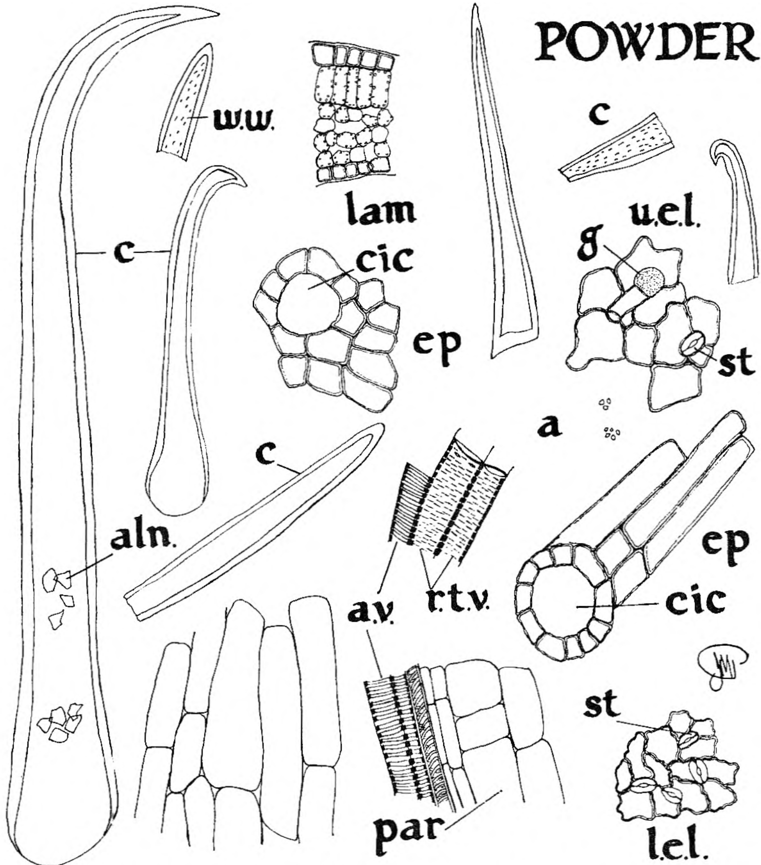

FIG. 5. Powder of *Symphytum officinale* L. All × 100. a, starch; aln., allantoin crystals; a.v., annular vessel; c, covering trichome; cic, cicatrix; ep, epidermis; g, glandular trichome; lam, lamina; l.e.l., lower epidermis lamina; par. parenchyma; r.t.v., pitted tracheidal vessel; st, stoma; u.e.l., upper epidermis lamina; w.w., warty wall.

80 μ wide, of straight unicellular trichomes with acute apices, with either thick smooth or warty cellulose walls; also fragments up to 60 μ wide, of unicellular trichomes with cellulose walls and hooked, acutely pointed apices. Both types may occur on any epidermal surface. Fragments of the *upper interneural epidermis* in surface view show cells with sinuous anticlinal walls occasionally with *anisocytic stomata*, also covering and *glandular trichomes*, with a unicellular stalk and unicellular sub-spherical

head; also fragments of *lower interneural epidermis* exhibit strongly wavy-walled cells with numerous anisocytic stomata and covering trichomes. Portions of *transverse sections of lamina* up to 100 μ wide, showing a single palisade and several layers of spongy mesophyll, both containing chloroplasts. Epidermal fragments of the midrib and petiole occur frequently, often showing large cicatrices. Fragments of vessels and tracheidal vessels are lignified and exhibit annular and pitted thickening. Occasional starch grains from the endodermis, allantoin crystals in the base of trichomes and mucilage from epidermal and pericyclic regions are also present. Calcium oxalate and sclerenchyma are absent.

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ABSORPTION AND ELIMINATION OF GRISEOFULVIN FROM THE ALIMENTARY TRACT OF THE RAT

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A new method is described for determining griseofulvin in the alimentary canal of rats dosed orally with the antibiotic: slightly modified, the method was used for measuring faecal griseofulvin. Attempts have been made to correlate the appearance of griseofulvin in the blood stream with its disappearance from the alimentary canal and to assess how much of a single oral dose is absorbed. Lack of griseofulvin at its main absorption sites in the alimentary tract was not responsible for the decline in blood level 4 hr. after oral dosing, because substantial amounts of unabsorbed antibiotic were then still present. Faecal elimination of griseofulvin, as determined by the new assay, is much greater than reported earlier.

Over the past 2 years much has been learned of the usefulness of griseofulvin as an antibiotic for treating dermatophyte infections in man and domestic animals; nevertheless, our knowledge of the degree and mechanism of its absorption from the alimentary canal remains meagre. Bedford and others (1960) conducted studies on cats and concluded that absorption of griseofulvin from the duodenum was a self-limiting phenomenon. They reported also that only 5·4 per cent of a dose administered orally to rats was detectable in the tissues at any time and that only 16 per cent was eliminated in the faeces during the 24 hr. after administration.

Having allowed for some of the absorbed griseofulvin having been destroyed by the liver, the amount calculated as remaining in the faeces was still unexpectedly low, and we therefore viewed with suspicion the ether extraction procedure used in determining faecal griseofulvin and considered it worthwhile to investigate the use of other solvents. In addition we attempted to correlate the disappearance of the antibiotic from the gut with its appearance in the blood stream and to assess the degree of absorption of single doses.

Materials and Methods

Animals

Male albino rats of the WAG strain (140–160 g.) were used in all experiments. Animals in groups of six were given single oral doses of 10 mg. griseofulvin presented as a 1 per cent aqueous suspension in 0·5 per cent Tween 80.

Collection of Faeces

The groups of six rats were housed in cages, with grid floors, previously washed with hot water and then with acetone to remove fluorescent contaminants. The faeces, which dropped through the grid on to a washed tray, were collected at various times after dosing.

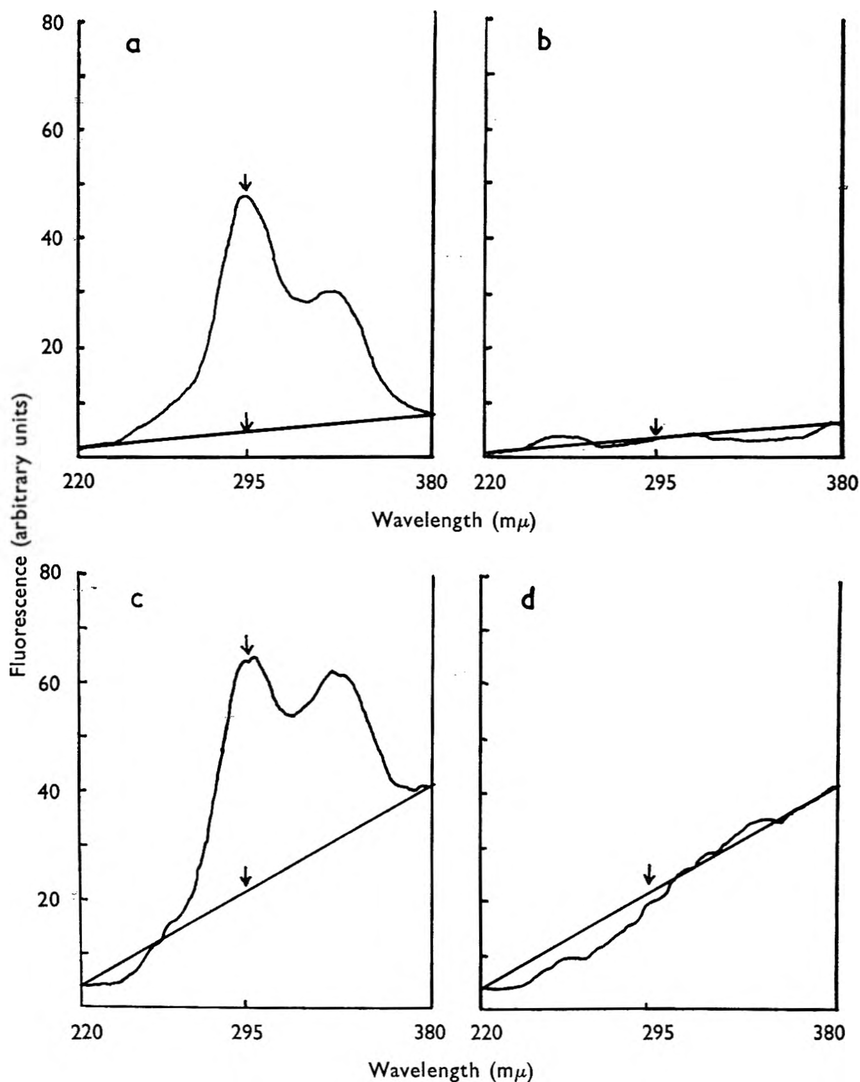

Fig. 1. (a) Scan of 1 per cent ethanol containing 0·5 μg. griseofulvin/ml.
(b) Scan of 1 per cent ethanol.
(c) Scan of small intestine extract dissolved in 1 per cent ethanol containing 0·5 μg. griseofulvin/ml.
(d) Scan of small intestine extract dissolved in 1 per cent ethanol.

(The different readings given in Fig. 1a and Fig. 2a by standard griseofulvin solution (0·5 μg./ml.) reflect changes in solution temperature or the sensitivity of the instrument.)

Estimation of Griseofulvin in Various Sections of the Alimentary Canal

Rats were killed and their entire alimentary tracts were removed. These were cut into four parts; oesophagus and stomach, small intestine, caecum, colon and rectum. The corresponding sections from all six rats in each group were bulked, finely divided with scissors and extracted thrice with acetone, once with 100 ml. and twice with 50 ml. The extracts were filtered (Whatman No. 4), and the filtrates were bulked and diluted with more acetone. The volume used was such that the solution obtained by evaporating 1 ml. of the final acetone solution and dissolving the residue in a suitable volume of 1 per cent ethanol gave a reasonable deflection on the most sensitive scale of a Farrand Spectrophotofluorometer.

This 1 per cent ethanolic solution was scanned through the activating range 380 to 220 mμ, with the analysing wavelength set at 450 mμ (uncorrected values). The intensity of the fluorescence derived from griseofulvin was calculated by joining with a straight line the scan readings at 380 and 220 mμ and subtracting the ordinate value of this straight line at 295 mμ from the total fluorescence at this wavelength. The fluorescence intensity so calculated was compared with that obtained similarly with a standard solution of griseofulvin.

The validity of this method of assessing the intensity of irrelevant fluorescence was verified by extracting tissues from control rats, scanning the extracts between 380 and 220 mμ and comparing the true blank value with that calculated by the straight line method. The two values obtained differed by not more than 2 arbitrary units, equivalent to a difference of approximately 4 per cent.

Typical scans for aqueous 1 per cent ethanol, 1 per cent ethanol containing 0·5 μg. griseofulvin/ml. and extract of small intestine with and without added griseofulvin are shown in Fig. 1.

The percentage recoveries of griseofulvin added in amounts of 10 or 60 mg. to various sections of alimentary tract were 94 $\pm$ 2·3 (four experiments) and 95 $\pm$ 4·0 (four experiments), respectively; the recoveries from the various sections were almost identical.

Estimations of Griseofulvin in Faeces

Distilled water sufficient to make a paste was added to the bulked faeces of each group, and the faecal paste was extracted once with 200 ml. acetone and thrice with 100 ml. The acetone extracts were filtered (Whatman No. 4) and bulked, and the volume was adjusted with acetone so that a reading was obtained on the most sensitive scale of the spectrophotofluorometer when 1 ml. of the final acetone solution was evaporated to dryness and the residue dissolved in 1 per cent ethanol. The concentration of griseofulvin in this solution was calculated by comparing the difference in fluorescence activities at activating wavelengths 380 and 295 mμ (analysing wavelength 450 mμ) with the activity of a standard griseofulvin solution calculated by the method previously described for the gut. This method of calculating irrelevant fluorescence derives from

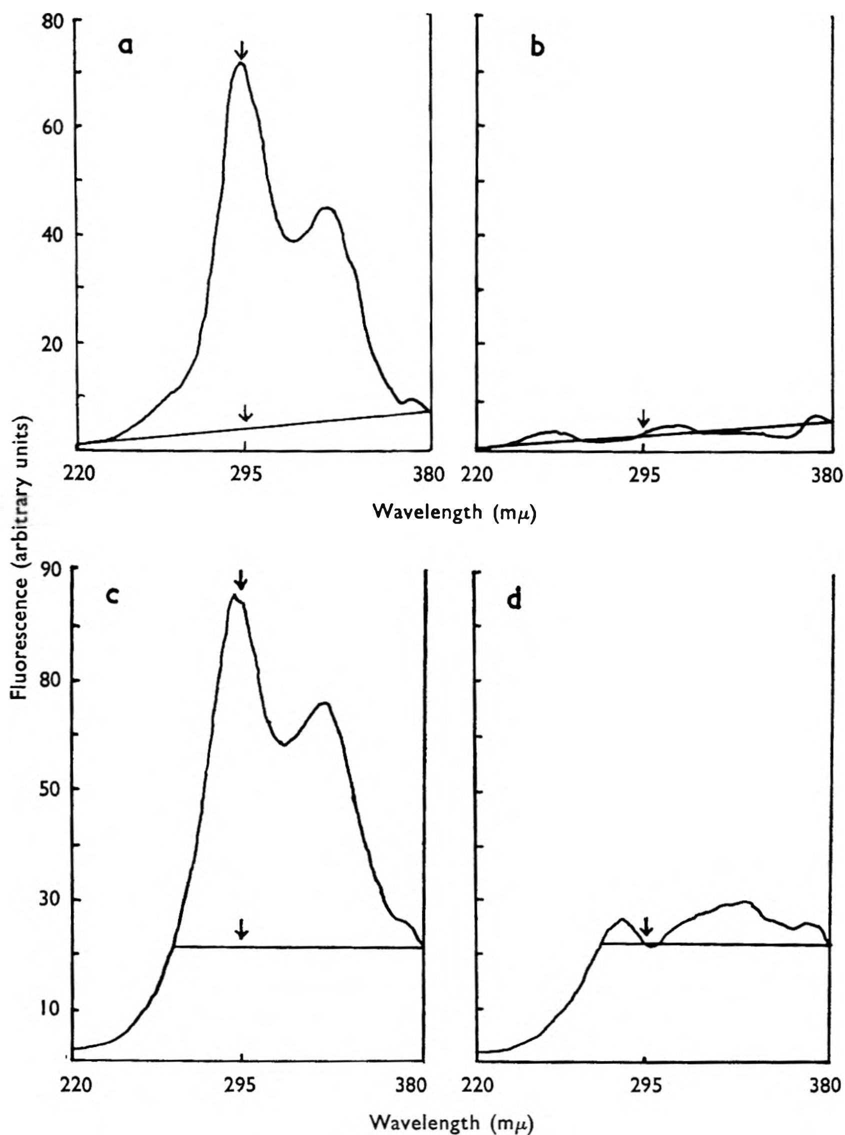

Fig. 2. (a) Scan of 1 per cent ethanol containing 0·5 μg. griseofulvin/ml.
(b) Scan of 1 per cent ethanol.
(c) Scan of faeces extract dissolved in 1 per cent ethanol containing 0·5 μg. griseofulvin/ml.
(d) Scan of faeces extract in 1 per cent ethanol.

(The different readings given in Fig. 1a and Fig. 2a by standard griseofulvin solution (0·5 μg./ml.) reflect changes in solution temperature or the sensitivity of the instrument.)

the fact that the fluorescence activities at 295 and 380 mμ were identical when faeces from controls were examined (Fig. 2).

The recovery of griseofulvin added in amounts of 30 or 60 mg. to the bulked faeces from six rats was 96 per cent.

Estimation of Griseofulvin in Blood

Blood samples were obtained from anaesthetised rats by direct cardiac puncture: heparin was added to prevent clotting (50 I.U. in 0·1 ml./3 ml. blood). The samples were assayed by the method of Bedford, Child and Tomich (1959).

RESULTS

Alimentary Distribution and Faecal Elimination of a Single Oral Dose

The grisecfulvin contents of different sections of the alimentary tract and of the faeces at various times after a single oral dose of 10 mg. are given in Table I.

The total recovery from the alimentary tract decreased with time. It will be seen that some griseofulvin spilled into the small intestine during oral administration. Four hr. after administration 91 per cent of the dose was recovered, this figure falling to 80 per cent at 8 hr. and 65 per cent at 18 hr. Twenty-four hr. after dosing a total of 55 per cent was recovered, of which 17·5 per cent was still in the alimentary tract and 37·5 per cent in the faeces. Faeces were not collected from animals killed 2 or 4 hr. after dosing.

TABLE I

GRISEOFULVIN PRESENT IN FAECES, BLOOD AND SECTIONS OF THE ALIMENTARY TRACT OF THE RATS AT VARIOUS TIMES AFTER A SINGLE ORAL DOSE (10 mg./rat)

	Percentage of dose recovered—hr. after administration							
	0	2	4	6	8	12	18	24
Stomach	92·5	40·2	23·4	18·8	4·9	0·4	0	0
Small intestine ..	7·4	56·0	52·1	41·5	30·5	17·0	17·7	10·8
Caecum	0	0	13·2	17·0	28·8	15·0	6·2	3·6
Colon and rectum	0	0	2·4	4·7	13·1	10·0	6·8	3·1
Faeces	—	—	—	0	2·7	26·6	34·3	37·5
Total	99·9	96·2	91·1	82·0	80·0	69·0	65·0	55·0
Blood level μg/ml.	—	1·51	2·27	1·09	0·81	0·02	—	—

Blood Levels after a Single Oral Dose of 10 *mg.*

Blood levels of other rats given single oral doses of 10 mg. are given also in Table I. The blood level reached a peak at 4 hr. and no griseofulvin could be detected 12 hr. after dosing.

DISCUSSION

Although experiments of Bedford and others (1960) on rats indicated that only a small percentage of a single oral dose of griseofulvin was

absorbed, nevertheless only 16 per cent could be recovered from the faeces in the first 24 hr. after dosing.

Because these results failed to account for the bulk of the griseofulvin, it was decided to investigate more fully its fate in the gut.

The results of the latest experiments, in which faeces and tissues were extracted with acetone, indicate that the amount of a single oral dose appearing in the faeces is much larger than had been suggested· In the first 24 hr. after a dose of 10 mg./rat the percentage of griseofulvin recovered in the faeces was 38. In similar experiments with 5 and 20 mg./rat the percentage recoveries were 30 and 64, respectively.

Immediately after oral administration most of the griseofulvin was present in the stomach, but after 2 hr. 56 per cent had passed through into the small intestine. At this time 96 per cent of the administered dose could be recovered from the alimentary canal, the concentration in the blood being 1·5 μg./ml.: at 4 hr. the corresponding figures were 91 per cent and 2·3 μg./ml. Thus up to 4 per cent had been absorbed in 2 hr. and 9 per cent in 4 hr. These values agree with the results of Bedford and others (1960), who recovered 2·6 and 5·4 per cent of the administered dose (100 mg./kg.) from the tissues of rats 2 and 4 hr., respectively, after dosing.

The amount of antibiotic recoverable from the alimentary canal continued to decrease from 4 hr. onwards at the same time as the blood level was falling. The decline in blood level cannot be attributed to the lack of alimentary griseofulvin, because substantial amounts were present throughout the entire length of the gut, 75 per cent being in the stomach and small intestine after 4 hr. Bedford and others (1960) have suggested that absorption of griseofulvin from the duodenum of the cat involves a self-limiting mechanism, and the results presented here indicate that something similar may occur in the rat.

The observation that griseofulvin continues to disappear from the alimentary canal simultaneously with the fall in blood level suggests that some destruction of griseofulvin may occur within the gut. Work is at present in progress to investigate this possibility.

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AUTOXIDATION, AND ITS INHIBITION, IN ANHYDROUS LANOLIN

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From Westbrook Lanolin Company, Argonaut Works, Laisterdyke, Bradford 4

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Long-term storage tests under good and bad conditions, and accelerated active oxygen method tests, have shown that autoxidative degradation of anhydrous lanolin can be satisfactorily inhibited for at least 32 months under good conditions or 2 years under bad conditions by 100 p.p.m. of butylated hydroxytoluene or butylated hydroxyanisole. Good results from other antoxidants, and disadvantages of some, are reported. Different lanolins are affected to different degrees, possible reasons being suggested, but autoxidation when uninhibited does not penetrate deeper than 1 cm. even after 2 years of adverse conditions; there is a fall in melting point and formation of up to 4·7 per cent of water-soluble substances including low-molecular weight acids. Absence of change in iodine value and rapid increase of acidity in darkness are contrary to previous reports.

ALTHOUGH oxidation of Anhydrous Lanolin (referred to hereafter as "lanolin"), its precursors and components has been previously studied,* most of this valuable work was based on accelerated oxidations or relatively short-term natural oxidation, or involved foreign lanolin refined by different processes to those used in this country, or reported changes only in acidity and peroxide value without distinction between surface and overall effects. It did not include long-term natural storage tests under good and bad conditions with a range both of antoxidants and concentrations in various lanolins. Also there are no reports about the depth to which autoxidation could penetrate, whether antoxidants inhibited other changes apart from those in acidity and peroxides, or whether there was similarity between the results of natural and accelerated oxidation of pharmaceutical lanolin.

Such investigations have therefore been made, using antoxidants recognised as innocuous (Ward, 1959; Min. of Food, 1954; Stat. Instr., 1958). Three experimental techniques have been used because (i) use of a single large container for storage tests limited the samples obtainable from the original, undisturbed surface and caused frequent changes of the air, (ii) use of many individual containers meant that they must be small, with potential errors because of differences in tightness of sealing and (iii) many antoxidant systems were involved.

* Clark and Thomas, 1949; Drummond and Baker, 1929; Freney, 1940; Gillam, 1948a; Gilmore, 1934; Horn, 1958; Horn and Ilse, 1956; Janecke and Senft, 1957a; Lewkowitsch, 1904; Lifschutz, 1924; Möllering, 1931; Muirhead and others, 1949; Nitschke, 1959; Peereboom, 1959; Ryberg, 1937; Salomone, 1930; Sandell, 1948, 1950; Windaus and others, 1941; Woodmansey, 1919.

Experimental

Storage in Closed Jars at Room Temperature

Lanolins used. (*a*) B.P., superfine; (*b*) and (*c*) B.P., Standard, (*d*) D.A.B. 6, Superfine, all of British manufacture*, and (*e*) D.A.B. 6 of German manufacture.†

Antoxidant systems used (*concentrations in p.p.m.*) (i) ±-α-tocopherol‡ 77, ascorbyl palmitate‡ 23; (ii) ±-α-tocopherol 20, ascorbyl palmitate 70, citric acid 10; (iii) butylated hydroxyanisole (B.H.A.)§ 100, propyl gallate¶ 30, citric acid 20; (iv) propyl gallate 500; (v) ±-α-tocopherol 100; (vi) B.H.A. 500; (vii) B.H.A. 200; (viii) B.H.A. 50; (ix) butylated hydroxytoluene (B.H.T.)* 500; (x) B.H.T. 200; (xi) B.H.T. 50; (xii) ±-α-tocopherol 50; (xiii) ±-α-tocopherol 20.

[Synergistic effects of citric acid (Janecke and Seft, 1957a; Stirton and others, 1945; Mattil, 1945), and system (iii) (Kraybill and others, 1948), were previously reported].

All but two of the antoxidants were dissolved in a little hot lanolin, the resultant concentrate being thoroughly mixed with the just-molten bulk. The exceptions, citric acid and propyl gallate, were dissolved in the minimum hot distilled water and stirred into the molten lanolin which was then homogenised. Each of the resultant lanolins was poured whilst still molten into amber glass jars of approximately 100 g. capacity. These were tightly closed by lacquered tin-plate screw caps with compressed paper wads, and packed into cardboard cases to exclude light completely. The storage temperature fluctuated between 4 and 25°.

Methods of examination. During the early stages of storage one jar from each test system was taken at monthly intervals for examination and analysis, later the intervals were increased. The surface of the contents of each jar was carefully removed with a flat scraper, 11 g. being taken. The average depth removed was approximately 7 mm., i.e. 18 per cent of the total depth. The surface portion and remainder of each sample were separately melted and stirred before analysis, the following tests being applied to both.

(1) Acid value: the method of the B.P. 1958.

(2) Iodine value: the method of the B.P. 1958 specified for Anhydrous Lanolin (iodine monochloride method).

(3) Saponification value: the method of the B.P. 1958, extending the time of reflux to 4 hr. and adding a little purified carborundum as boiling-aid.

(4) Peroxide value: the simplified Lea (1938) method, expressing results as ml. of N/500 thiosulphate per g. of sample.

* "Golden Dawn", Westbrook Lanolin Company.

† "Goldenes Vliess", Woll-Wäscherei und Kämmerei in Döhren bei Hanover.

‡ Roche Products Ltd.

§ "Tenox", Kodak Ltd., and "Embanox", May & Baker Ltd.

¶ "Progallin", Nipa Laboratories Ltd.

* "Shell Antiodixant T.P. 6335", Shell Chemical Co. Ltd.

(5) Melting point: the method of the B.P. 1958, but to avoid cold-working errors the cups were filled with molten lanolin and allowed to stand 24 hr. at room temperature before the determination.

(6) Colour: measured whilst molten by an EEL photoelectric colorimeter using blue and green filters. Results were converted by a calibration graph to equivalent Lovibond Yellow and Red units for a ¼ in. cell.

(7) Total cholesterol: the Liebermann-Burchard method, measuring the intensity of colour on an EEL photoelectric colorimeter with a red filter. A gravimetric precipitation by digitonin from the original lanolin (after conversion of esterified cholesterol to the free state) was used as

TABLE I

CHANGES IN SURFACE LAYER OF D.A.B. 6 SUPERFINE LANOLIN (BRITISH) DURING STORAGE IN PART-FULL CANS

	Original results	Results after 2 years with antoxidant system:						
		Control	(i)	(ii)	(iii)	(iv)	(v)	(vi)
Acid value	0·24	9·46	5·42	3·64	0·30	0·38	6·64	0·30
Saponification value	97·5	107·0	104·0	106·8	98·0	98·0	111·3	96·0
Ester value	97·3	97·5	98·6	103·2	97·6	97·5	104·7	95·6
Peroxide value	11·0	166·0	157·0	149·0	12·0	9·0	237·0	12·0
Iodine value	28·0	24·4	22·9	25·4	27·0	29·1	24·0	26·9
Unsaponifiables, per cent	51·0	44·7	47·6	42·9	52·1	49·9	43·0	53·4
Total saponifiables, per cent	50·0	52·1	53·2	56·3	50·0	52·0	56·9	48·4
Total recovery, per cent	101·0	96·8	100·8	99·2	102·1	101·9	99·9	101·8
Total cholesterol, per cent (Liebermann-Burchard)	20·8	14·0	12·1	15·5	18·9	18·3	13·2	18·3
Total cholesterol, per cent (Digitonin)	20·8	13·3	14·2	13·5	19·5	17·2	10·3	19·6
Melting point (°C)	38·2	35·7	37·8	36·8	38·0	37·4	37·6	37·7
Colour	4·6Y: 0·4R	4·1Y: 0·4R	4·8Y: 0·5R	4·2Y: 0·6R	3·3Y: 0·4R	6·9Y: 0·8R (originally 6·8Y: 0·9R)	3·3Y: 0·4R	4·1Y: 0·6R

calibration. These two methods are affected by other steroids or oxidative degradation products which may be present, therefore the results given are not strictly accurate.

The full range of tests was made on samples (*c*) and (*d*) and since little, if any, change appeared to be occurring in other characteristics, acidity and peroxide values only were determined on most of the older samples. With samples (*a*) and (*b*) tests were further confined to the surfaces.

Additional observations were made about the effect of surface autoxidation on permanganate tests and the development of tallowy odour and tough skin.

Storage at Room Temperature in Part-full Cans

Lanolins and antoxidant systems used. Lanolins (*c*) and (*d*) and Systems (i) to (vi) were used.

Method. Standard cylindrical tin-plate cans of 5 kg. capacity were filled with molten lanolin to the 4 kg. level only, leaving an abnormally large air space to represent bad storage conditions. The filled cans with lids applied in the normal way were stored undisturbed for 2 years as previously described, before being examined.

Examination of the surface. The lanolin within a lightly-inscribed circle of 3 in. (76 mm.) diameter was carefully removed to a depth of $\frac{1}{8}$ in. (3·2 mm.) by a spatula and melted and mixed. The tests previously described were applied and in addition, ester values were calculated and

TABLE II

PENETRATION OF AUTOXIDATION IN D.A.B. 6 SUPERFINE LANOLIN (BRITISH) DURING STORAGE IN PART-FULL CANS

Depth of sample (in.)	Original acid value	Acid value after 2 years with antoxidant system:						
		Control	(i)	(ii)	(iii)	(iv)	(v)	(vi)
0–$\frac{1}{8}$	0·24	9·46	5·42	3·64	0·30	0·38	6·64	0·30
$\frac{1}{8}$–$\frac{1}{4}$	—	0·82	0·98	0·78	0·30	0·30	0·82	0·28
$\frac{1}{4}$–$\frac{3}{8}$	—	0·40	0·42	0·44	—	—	0·36	—
$\frac{3}{8}$–$\frac{1}{2}$	—	0·29	0·30	0·40	—	—	0·29	—
$\frac{1}{2}$–$\frac{5}{8}$	—	0·29	0·30	0·29	—	—	0·29	—
$\frac{5}{8}$–$\frac{3}{4}$	—	—	—	0·30	—	—	—	—

unsaponifiables and total saponifiables determined. The results for unsaponifiables were corrected for any soap content resulting from incomplete extraction and the corrections added to the figures for total saponifiables. The latter were determined and not calculated by subtraction of the unsaponifiables from 100 per cent.

Examination of the lower layers. After removal of the upper $\frac{1}{8}$ in., successive depths of $\frac{1}{8}$ in. were removed from the same area. Each

TABLE III

PENETRATION OF AUTOXIDATION IN D.A.B. 6 SUPERFINE LANOLIN (BRITISH) DURING STORAGE IN PART-FULL CANS

Depth of sample (in.)	Original peroxide value	Peroxide value after 2 years with antoxidant system:						
		Control	(i)	(ii)	(iii)	(iv)	(v)	(vi)
0–$\frac{1}{8}$	11	166	157	149	12	9	237	12
$\frac{1}{8}$–$\frac{1}{4}$	—	33	41	26	12	10	42	13
$\frac{1}{4}$–$\frac{3}{8}$	—	13	8	12	—	—	12	—
$\frac{3}{8}$–$\frac{1}{2}$	—	8	10	9	—	—	12	—
$\frac{1}{2}$–$\frac{5}{8}$	—	9	10	11	—	—	—	—
$\frac{5}{8}$–$\frac{3}{4}$	—	—	—	—	—	—	—	—

portion was separately melted and mixed before being tested for acidity and peroxide value. Removal of portions was continued until there was no further change in characteristics.

RESULTS AND DISCUSSION

Many of the graphs of results from storage in closed jars at room temperature showed large fluctuations. Whilst peroxide values may reach a maximum and then decrease as the rate of decomposition of peroxides exceeds the rate of formation (Paschke and Wheeler, 1944), it is almost inconceivable that a value should re-approach the original one whilst oxidisable matter still remains, or that decomposed cholesterol should be regenerated. We have assumed that sudden falls in a graph resulted from lack of oxidation because of a high degree of sealing of some jars. With efficient antoxidant systems instances of sudden fluctuations were few and effects were slight, and changes in all chemical and

physical characteristics were satisfactorily inhibited for the duration of the tests.

Acid value: peroxide value. Increases in these values were the most significant effects of autoxidation, Figs. 1–4 typify the results obtained. Other comparative tests followed a similar trend although the increases were smaller. In many instances, including samples which had suffered negligible surface oxidation, there appeared to be a very slight increase

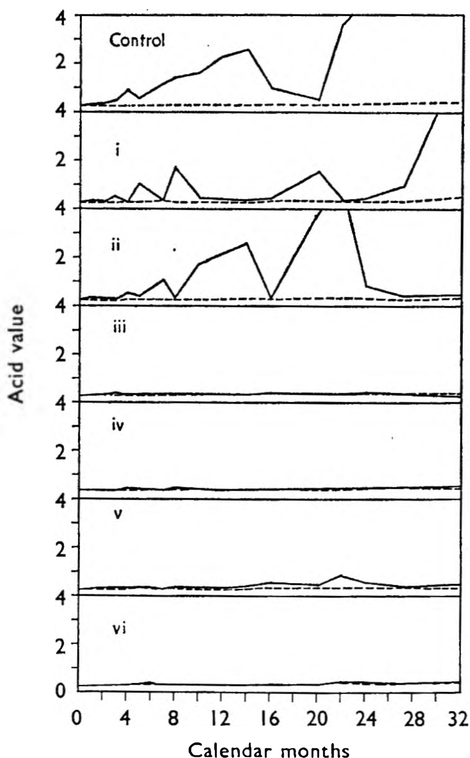

FIG. 1. Change in acid value of lanolin (sample (d)) stored in jars.
—— Surface layer - - - - Underlying bulk.

in acid value of the bulk after long storage, suggesting a gradual hydrolysis of the lanolin esters.

Iodine Value. Changes were negligible, even in highly oxidised samples, and are therefore not reported. These findings contradict those of Salomone (1930) but may be explained by other work (Bergström, 1943; Gillam, 1948b; Gunstone and Hilditch, 1945; Horn, 1958; Horn and Ilse, 1956; Keller and Weiss, 1950; Weiss and Keller, 1950).

Saponification value: ester value. Increases in both occurred, indicating esterification or condensation of many of the acids produced by autoxidation similar to previously-reported results of accelerated oxidation. The fall in total recovery, i.e. unsaponifiables plus total saponifiables, from the normal value of over 100 per cent indicates that up to 4·7 per

cent of water-soluble by-products may be present in highly oxidised lanolin. Some of these by-products are indicated to be acids of relatively low molecular weight since an aqueous extract of the oxidised lanolin invariably had a low pH.

Melting point. Although badly oxidised surfaces of lanolin consisted of tough skins the melting points found were lower than those of the corresponding bulks (Muirhead and others, 1949; Clark and Kitchen, 1960). The decreases found initially being small, are not reported.

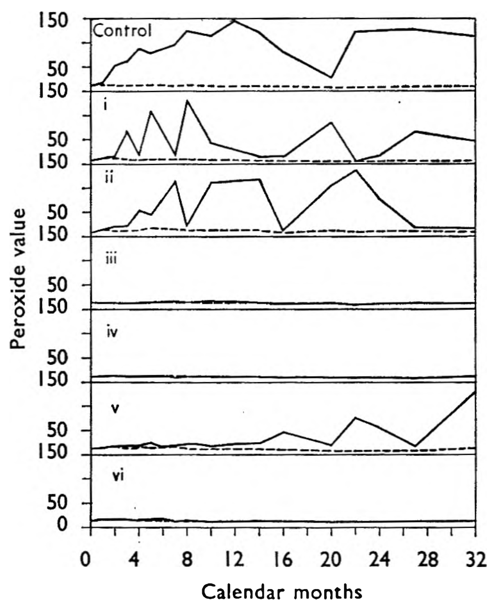

FIG. 2. Change in peroxide value of lanolin (sample (d)) stored in jars.
—— Surface layer - - - - Underlying bulk.

Colour. Bleaching of autoxidised surfaces was found, and is normal although some lanolins of German manufacture have been known to darken (also reported by Salomone, 1930). The addition of the larger concentrations of propyl gallate brought an immediate darkening in colour.

Cholesterol content. The autoxidative degradation found agrees with previous work except that of Norcia (1958). In some instances the lower bulk of a sample showed a very slight decrease (of doubtful significance) in cholesterol content.

Permanganate test. The effect of autoxidation upon a permanganate test as specified for lanolin by several pharmacopoeias has already been studied (Sandell, 1950). Initially permanganate tests were made at irregular intervals, and the results showed that the surface of lanolin which originally complied began to fail the test (the D.A.B. 6 method was used) in 2 to 3 months, the lower bulk remaining unaffected. Efficient antoxidants prevented formation of permanganate-reducing substances

until other chemical changes became detectable. Sandell (1950) reported that the incorporation of propyl gallate in lanolin adversely affected the permanganate test because of aqueous solubility. We confirmed this effect and found it to extend also to B.H.A. and citric acid, the initial effect of B.H.A. being slight but increasing with age. B.H.T. and α-tocopherol did not have the effect.

Odour and skin formation. A tallowy odour and hard skin were found only in samples which showed significant chemical changes. The first change in odour was apparent after 3 months, and the first definite skinning after 5 months.

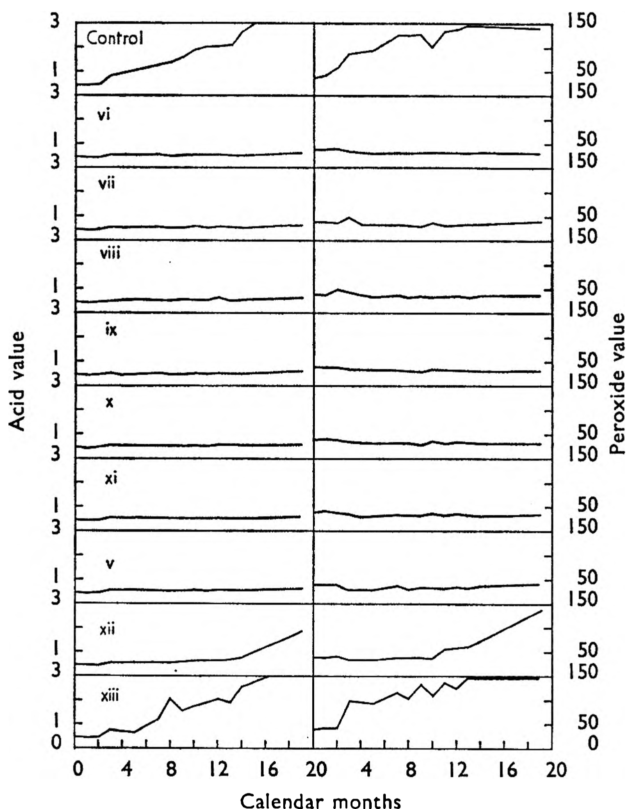

FIG. 3. Changes in acid value and peroxide value of lanolin stored in jars (sample (*a*); surface layer only).

Light. Contrary to Möllering's (1931) findings the results show that complete absence of light does not prevent autoxidative increase in acid value.

Differences in rates of oxidation. Differences in rates of oxidation between different types of crude wool fat have been noted previously. With refined lanolin, the peroxide and acid values in particular showed that the samples differed greatly in their response to both oxidation and

antoxidants, even though processes used in refining the British lanolins had differed in only minor ways. Possible reasons are: (i) Differences in content of trace metals such as copper and manganese which can act as oxidation catalysts (King and others, 1933b; A.O.C.S., 1945). Although manganese compounds have been isolated from wool fat (Truter and Woodford, 1954) the amounts in high quality pharmaceutical lanolin are minute. (ii) Variation in traces of phosphorus compounds. These have been found in lanolin by Drummond and Baker (1929), Janistyn

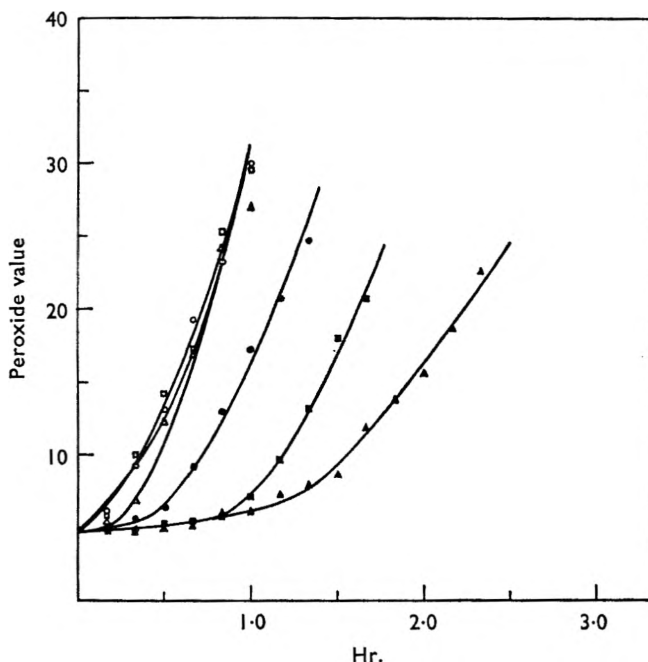

FIG. 4. Active oxygen method tests on lanolin. The antoxidant effect of ±-α-tocopherol.
○ control. □ 10 p.p.m. △ 40 p.p.m. ● 100 p.p.m. ■ 200 p.p.m. ▲ 500 p.p.m.

(1940a,b), and Janecke and Senft (1957b), and the effect of phosphatides on oxidation of oils has been reported by Braae (1958). (iii) Existence of natural antoxidants in raw wool fat, variable quantities being carried through the refining processes into the finished lanolin. (iv) The catalytic effect of peroxides already in the lanolin. The order of susceptibility to oxidation found was not apparently related to the original peroxide values. (v) Differences in previous history of the lanolins. Although raw wool fat on the fleece oxidises extensively the principal oxidation products are removed during refining, but traces of some substances may remain and promote subsequent oxidation to varying degrees. (vi) Differences in pH. We have found that minor details in processing which affect the pH of an aqueous extract of the finished lanolin may also affect its general stability.

EXPERIMENTAL. II

Active Oxygen Method. The active oxygen method (A.O.M.) (Wheeler, 1932; King and others, 1933a) involves passing air at a controlled rate through the sample at a controlled temperature. In the present work progress of oxidation was followed by determining the increase in peroxide value.

The apparatus comprised a liquid paraffin heating bath surrounding a 1 l. three-necked reaction flask; a mechanical pump which drew purified air through a pre-heating coil in the paraffin bath, through the molten lanolin in the flask, and finally through a flowmeter. The rate of air

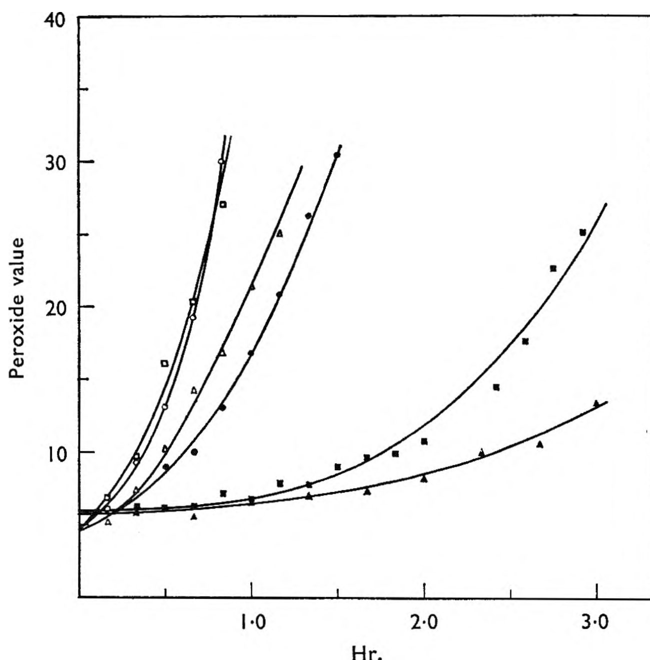

FIG. 5. Active oxygen method tests on lanolin. The antoxidant effect of butylated hydroxyanisole.

○ control. □ 1 p.p.m. △ 5 p.p.m. ● 10 p.p.m. ■ 40 p.p.m. ▲ 100 p.p.m.

flow was controlled at 180 ml./sec. although small variations in rate did not appear to affect significantly the rate of oxidation.

A test involved first heating the bath to about 92°. Meanwhile 200 g. of lanolin were separately heated to 90° and any antoxidant, dissolved in 1 ml. of isopropanol, was added immediately before pouring the hot lanolin into the reaction flask whereupon the flow of air was started without delay. A control experiment showed that the isopropanol evaporated within 2 or 3 min. and had very little effect upon oxidation. The contents of the flask were maintained at 90° throughout the test, samples being removed at regular intervals whilst momentarily interrupting the air flow.

The temperature of 90° was adopted after preliminary experiments, the results of which demonstrated the normal accelerating effect of heat upon the rate of oxidation, particularly between 80° and 90°.

Type of lanolin used. Lanolin similar to (*d*) in Part I was used throughout, but because of the amount of work and time involved, samples from different production batches were unavoidably necessary. Since it was found that different batches of lanolin varied in susceptibility to oxidation, control tests were used in choosing batches as similar as possible in behaviour. Experiments made with different batches could

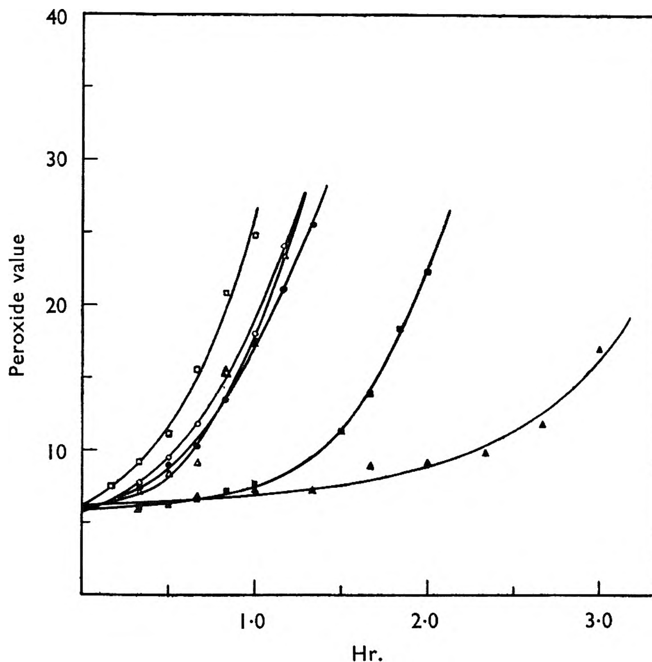

FIG. 6. Active oxygen method tests on lanolin. The antoxidant effect of butylated hydroxytoluene.
○ control. □ 1 p.p.m. △ 5 p.p.m. ● 10 p.p.m. ■ 40 p.p.m. ▲ 100 p.p.m.

not, however, be strictly correlated but series of tests involving a range of concentrations of one antoxidant were always completed on the same lanolin.

Antoxidant systems used (*concentrations in p.p.m.*). ±-*α-tocopherol*, 10–500. Also 50–100 plus ascorbyl palmitate (50–200) and citric acid (20–100). B.H.A. 1–40. Also 10 plus citric acid (20–50). B.H.T. 1–40. Also 5–100 plus citric acid (20–100). Ascorbyl palmitate, 50–2000. Citric acid, 20–100.

RESULTS

Graphs prepared from the results showed very few clearly defined induction periods, most conforming to a basic pattern. Accordingly only typical examples are given in Figs. 4–6.

Discussion

Since the efficiencies of individual antoxidants were not linearly related to concentration no definite order of efficiency could be established. In general, however, B.H.A. was the most efficient single antoxidant, B.H.T. and propyl gallate almost as good, but α-tocopherol and ascorbyl palmitate much inferior although useful. Ascorbyl palmitate, and particularly citric acid, in addition to having antoxidant properties of their own, strongly augmented the effects of other antoxidants but, whilst in some combinations with α-tocopherol and B.H.A. there appeared to be true synergism, in others the effect may have been merely additive. Ascorbyl palmitate actually reduced peroxides already present in the lanolin at the expense of darkening the colour; at low concentrations ($\leqslant$200 p.p.m.) it had a pro-oxidant effect (Ward, 1959; Baltes, 1955; Baltes and Volbert, 1955), as did also B.H.T. ($\leqslant$5 p.p.m.).

Concerning all the antoxidants tested, the same approximate order of efficiency prevailed throughout, and where results overlapped they agreed fairly well with previous work. We found, however, ascorbyl palmitate and citric acid to be more efficient than did Janecke and Senft and also found a more marked superiority of B.H.A. over α-tocopherol.

Since B.H.T. had almost the highest efficiency and was free from any disadvantages in respect of solubility, colour, permanganate test and acidity, it is indicated to be the most suitable general-purpose antoxidant for lanolin, a concentration of 100 p.p.m. being satisfactory.

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NEW APPARATUS

A NON-CORRODIBLE VALVE FOR USE IN AUTOMATIC CONTROL OF FLOW OF PHYSIOLOGICAL FLUIDS

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A valve made from non-corrodible materials, with the method of construction, relevant measurements, order of assembly and final adjustments is described.

IN the apparatus first described by Schild (1946) for the automatic filling and emptying of an isolated tissue bath, the flow of fluid to and from the bath was controlled by compression of rubber tubing under the armature of a telephone relay so that when the solenoid was energised, the pressure on the tube was released and the fluid was allowed to run. Other methods have since been used for the automatic control of fluid flow. In this laboratory we have designed an automatic control valve for the flow of physiological fluids. The valve is constructed from an oil dilution valve A.M. 5U/3013 type FAW/A/221 and is controlled by a current source of 24 V. The valve consists of plastic and nylon components which do not contaminate the fluids and which have not shown any evidence of corrosion. It has been used for two years during which it has required no adjustments except periodic cleaning. The interior of the valve consists of a barrel and plunger which can be separated for cleaning. Inlet and outlet tubes are screwed into the barrel. Fig. 1.

The barrel was constructed from a 2-in. length of $\frac{3}{4}$ in. diameter perspex rod. A hole was first drilled through the long axis with a letter "D" drill (0·246 in.). The hole was then increased in diameter to a depth of $\frac{1}{2}$ in. and tapped $\frac{1}{4}$ × 19 B.S.P. A hole 1/16 in. in diameter was then drilled from the angled shelf made by the drill to meet the exterior of the barrel approximately $\frac{3}{4}$ in. from its end. This vent is necessary to control changes in air pressure caused by movement of the plunger in the barrel. The internal diameter of the other end of the barrel was correspondingly enlarged and counterbored with a flat ended drill to make the final depth 5/16 in., and threaded $\frac{1}{4}$ × 19 B.S.P. A hole for the outlet tube was made with a No. 26 drill and threaded 2 B.A., the centre being $\frac{1}{2}$ in .from the end of the barrel and in line with the vent.

The plunger was made from $1\frac{1}{4}$ in. of $\frac{1}{4}$ in. diameter P.T.F.E. rod. The diameter of one end was reduced to 0·225 in. for a length of $\frac{1}{2}$ in. and the tip was turned to form a 60° inclusive point. The other end of the plunger was drilled to a depth of $\frac{5}{8}$ in. and threaded to 4 B.A. to take the plunger screw.

The plunger screw was made from $1\frac{1}{8}$ in. of 7/32 in. diameter brass rod. One end of the rod was turned down to a distance of $\frac{7}{8}$ in. and threaded to 4 B.A. This was later secured to the plunger by a 4 B.A. half nut

suitably reduced in external diameter. At a distance of $\frac{1}{8}$ in. from the other end of the rod a 3/32 in. hole was drilled across the diameter.

The inlet and outlet tubes were made from nylon rod. The inlet tube was constructed by turning down one end of $1\frac{1}{8}$ in. of nylon rod $\frac{3}{4}$ in. diameter to a distance of 5/15 in. and threading it to $\frac{1}{4} \times 19$ B.S.P. This end was then centre drilled with a No. 1 centre drill to form the valve seating which at this stage had a diameter of 0·225 in. This end of the inlet tube was then skimmed square until the diameter of the valve seating

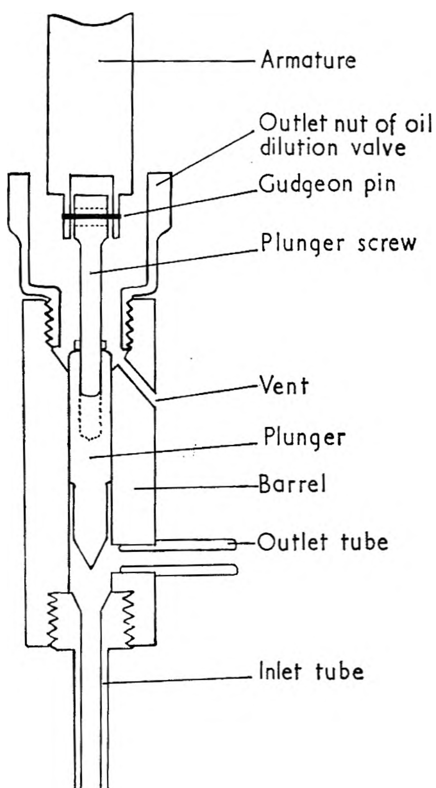

FIG. 1.

was 0·2 in. A hole was then drilled with a No. 30 drill through the long axis of the rod. The construction of the inlet tube was completed by screwing its threaded end into the barrel and turning both between the chuck and a live centre until the outside diameter was reduced to 3/16 in. For the outlet tube approximately $\frac{5}{8}$ in. length of 3/16 in. diameter nylon rod was drilled throughout its long axis with a No. 30 drill. It was threaded to 2 B.A. for a distance of 3/16 in. from one end for attachment to the barrel.

Before fitting the valve it was necessary to modify the oil dilution valve 5U/3013 type FAW/A/221, the outlet nut of which was drilled out to $\frac{3}{8}$ in. to give clearance for the plunger screw. In order that the plunger

screw could be connected to it the armature was also modified by removing the washer and drilling a hole ¼ in. diameter and ¾ in. deep in the centre of the exposed end; it was then turned down to 5/16 in. for a distance of ¼ in. A hole 1/16 in. in diameter was drilled ⅛ in. from the end of the armature to take the gudgeon pin.

Before assembly the inlet tube was removed from the barrel and the latter was reamed out to 0·25 in. The plunger with the plunger screw in position was carefully lapped into the barrel by hand to give a slide fit, using fine carborundum paste. The hole in the plunger screw head was aligned within the armature and secured in position by a silver steel gudgeon pin. The loose fit acts as a universal joint to allow the plunger to move freely in the barrel. The oil dilution valve was then reassembled with the plunger protruding from the outlet nut. The inlet tube was again screwed into the barrel until it just touched the flat base of the hole. The valve seating is liable to distortion if the inlet tube is screwed too firmly. The barrel was then placed over the plunger and screwed to the outlet nut. It is usually necessary to remove the barrel several times to adjust the position of the plunger on the plunger screw until the maximum flow of fluid is obtained.

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PHARMACOPOEIAS AND FORMULARIES

The National Formulary of the United States of America, Eleventh Edition, 1960*

Reviewed by K. R. Capper

The National Formulary of the United States for over 50 years has provided standards for drugs and preparations of drugs additional to those in the United States Pharmacopeia. During this period it has become accepted internationally as well as nationally as an important book of standards. To an extent which has increased considerably during the last twenty years, those drugs which have achieved pharmacopoeial status in the major national pharmacopoeias are also those which are used in all countries. It is in secondary publications, such as the National Formulary and the British Pharmaceutical Codex, that there may still be found those medicines which have a regional popularity, and the National Formulary has always contained a fair number which have been little used in Great Britain. In recent editions those vegetable drugs and their preparations based on the indigenous flora of the United States, and which have seemed to us as characteristic of a picturesque era in American pharmacy, have largely disappeared from the N.F., and, of the few remaining in the N.F.X., Grindelia, Lobelia and Hydrastis are in the list of omissions from the eleventh edition. Amongst the 207 deleted monographs are those for all the hypophosphites and all the glycerophosphates; several barbiturates have been omitted, but as an example of differences in medical practice even with these drugs, cyclobarbitone, recently added to the B.P., has been dropped from the N.F., while barbitone, now little used in this country, has been retained.

More than 250 new monographs have been added, about half this number being for drugs and the remainder for their preparations. Of the drugs, half are those which have been omitted from the United States Pharmacopeia, while the other half, about 60 in all, are new in the sense that they have not previously been officially recognised in the United States. As a therapeutic list it is not impressive, as indeed it cannot be, because the U.S.P. and the N.F. are published more or less simultaneously and the Pharmacopeia has first choice. Thus, the only "new" antibiotics in the N.F. are Oleandomycin (as the phosphate and as the triacetyl derivative) and Gramicidin, whilst to the U.S.P. has been added Erythromycin, Novobiocin, Nystatin and Phenoxymethylpenicillin. Additions to other therapeutic groups in the National Formulary are similarly incomplete unless regarded as supplementary to the U.S.P. additions. Some of the additions are not yet in general use in Great Britain but most are. They include the hypnotics Glutethimide, Ethinamate and Methyprylon, the analgesics Paracetamol (under the title Acetaminophen), Salicylamide and Levorphanol Tartrate, the anticoagulant Diphenadione

* Published by the American Pharmaceutical Association, Washington, D.C., U.S.A. Pp. xxxii + 531 (including index). $9.00.

and the oestrogen Chlorotrianisene *inter alia*. In view of the comments which are made about the time which it takes in this country for a new drug to become official, it is interesting to be able to find out how long it takes in the United States. The publication New and Non-official Drugs gives dates for the introduction into the United States of the drugs it describes, and from these it can be seen that, in most instances, the drugs have been in use for more than five years, sometimes very much more, before obtaining official recognition in either book.

In general style, the monographs are similar to those in the previous edition but a two-column format has been adopted. This format is not uncommon in United States books, more so than in those published in this country, and it is probably a nationalist prejudice to say that one does not care for it very much. The type of specification and the information given is similar to that in B.P. monographs except that the therapeutic category of each drug is given. Anticipating the B.P., apothecaries' doses are omitted. The number of spectrophotometric assays has been increased, and these must be carried out using reference standards for comparison. The reference standards obtainable from the United States Pharmacopeia Authorities together with those obtainable from the American Pharmaceutical Association form an impressive list. The use of a reference standard is almost essential if consistent results are to be obtained in different laboratories and it seems likely that absolute methods now in the B.P. and B.P.C. will be replaced and that reference standards will have to be provided in this country. For a small number of drugs in the National Formulary, for example, Azacyclonol and Sitosterols, infrared absorption is used for identification. Assays based on titration in non-aqueous media are specified for a number of drugs, including Aminoacetic Acid, Noscapine Hydrochloride and Isoprenaline Sulphate, each of which is titrated against perchloric acid in glacial acetic acid, and for Barbitone and some other barbiturates which are titrated in dimethylformamide against lithium methoxide. Reserpine in rauwolfia is determined by a method similar to that recently recommended by a panel of the Joint Committee on Methods of Assay of Crude Drugs. A variation which may produce significant differences in the stated content of the drug, is in the method of extraction. In both methods a Soxhlet extraction with ethanol is used but the panel found that a 4-hr. extraction was not always adequate to remove the alkaloids completely and it recommended a preliminary maceration with ethanol acidified with dilute acetic acid. The National Formulary method does not include this.

From the point of view of pharmaceutical practice, there is an interesting extension of information in the recommended methods for preparing ophthalmic solutions. A general recommendation is that all eye-drops should be buffered by the addition of 2 per cent boric acid except where this is incompatible, e.g. with pilocarpine, sulphonamides and fluorescein sodium. The methods recommended for the extemporaneous preparation of eye-drops differ to some degree according to whether they are to be applied where the corneal epithelium has been damaged or where it is not damaged. Where there is damage the solutions should be distributed

in small containers which should be used for one patient only; the eye-drops in the closed container and an eye-dropper should be separately packed and autoclaved, and no preservative should be added. Where there is no damage, the eye-drops may be made with stock vehicles containing a suitable preservative and sterilised either by autoclaving or by boiling for at least 30 min. The final containers should be similarly sterilised and so should equipment used to make the eye-drops. Once the stock vehicle has been opened, the vehicle should either be discarded or re-sterilised if kept longer than 24 hr. It is pointed out that the criteria for the use of ophthalmic solutions in the home and in the surgery or clinic are very different as in the latter circumstances contaminated solutions have resulted in the transfer of organisms from one patient to another.

Dr. Justin Powers, Chairman of the National Formulary Committee, has for five editions carried a major responsibility for producing this book. The international repute of the National Formulary is a tribute to his success and one which it can be certain will be maintained by his successor. It is, however, not only as a very distinguished pharmacist that he is esteemed in many countries besides his own, but also as a most kindly, approachable, and helpful person.

BOOK REVIEW

BENTLEY AND DRIVER'S TEXTBOOK OF PHARMACEUTICAL CHEMISTRY. 7th Edition. Revised by R. E. Driver. Pp. ix + 728 (including Index). Oxford University Press, London, 1960. 63s.

Although the general arrangement of this new edition follows the lines of its predecessors the text has been extensively revised, much of it has been rewritten and some new material has been included.

The book as before is divided into three parts: analytical methods, inorganic and organic. Two new chapters have been added to Part I, one a short account of organometallic complexes in analysis and the other an account of measurement of radioactivity. The extension of the section on ultra-violet absorption has improved the chapter on photometric methods.

Part II now begins with elementary but clear and concise accounts of atomic nuclei and radioactivity and periodicity and valency. The remaining chapters which describe the more important inorganic compounds used in pharmacy follow the lines of previous editions except that they have been revised to exclude compounds no longer described in the *British Pharmacopoeia.*

Part III which comprises the organic section has been improved by being extensively rewritten on more general lines. It is perhaps a pity that the author did not take the opportunity to go further in an attempt to present the theoretical aspects of the subject as an integral part of the description of the behaviour of organic compounds. Where possible medicinal compounds recognised by the current Pharmacopoeia are used as examples of each organic type.

A welcome feature is the increased space given to descriptions of synthetic medicinal chemicals. This was previously dealt with in one chapter and, has now been extended to twelve chapters each dealing with a different chemical type. The sections dealing with proteins, hormones, vitamins and antibiotics have also been improved by extension.

It is comparatively easy to criticise this work on the basis of its omissions. This arises from the misconception that one volume could adequately cover all aspects of pharmaceutical chemistry. The student who reads either for a diploma or a degree is required to have a knowledge of the principles underlying analytical methods, classical physical chemistry, general and inorganic chemistry and the principles of organic chemistry; it is asking too much that one textbook should adequately cover all these topics. The author obviously realising this has had to in some instances curtail and in others omit certain aspects of the subject. Perhaps in future editions the author will consider the inclusion of such topics as the factors influencing drug action, structure activity relationships and general chemotherapy. This would be useful in the light of the requirements for the three year diploma students.

It might also be helpful if instead of including the occasional reference, each chapter was followed by suggestions for further reading.

In spite of the above criticisms this new edition of "Bentley and Driver" will still continue to be of great use to students, particularly diploma students of pharmaceutical chemistry. The book is beautifully printed on fine quality paper.

N. J. HARPER.

LETTER TO THE EDITOR

Histidine Decarboxylase in Rat Hepatoma

SIR,—As part of the programme designed to test the theory of Kahlson (1960) that the histamine-forming capacity of rat tissues is related to the processes of growth, regeneration and repair, we have studied the growth of a transplantable rat hepatoma. This was kindly supplied by Professor A. Haddow of the Chester Beatty Research Institute, London, and has already been shown by Mackay, Marshall and Riley (1960) to possess a high histidine decarboxylase activity.

TABLE I

COMPARISON OF THE PROPERTIES OF HISTIDINE DECARBOXYLASE FROM THREE LOCATIONS IN THE RAT

Location of enzyme	Weight of tissue (g.)	Histidine decarboxylase			5-Hydroxy-tryptophan decarboxylase (μg.)
		Activity (μg.)	Optimal pH	Benzene required	
Hepatoma (day 10)	0·4	474	6·5	No	3·0
Foetal liver (day 15)	0·03	68	6·5	No	0·1
Adult liver	7·0	70	8·0	Yes	910·0

Female rats of the August strain and of the same age were implanted subcutaneously with the hepatoma and determinations of the histamine-forming capacity of the tumour were made at intervals using the method of Waton (1956) as modified by Telford and West (1961a). The tumour enzyme showed an optimal

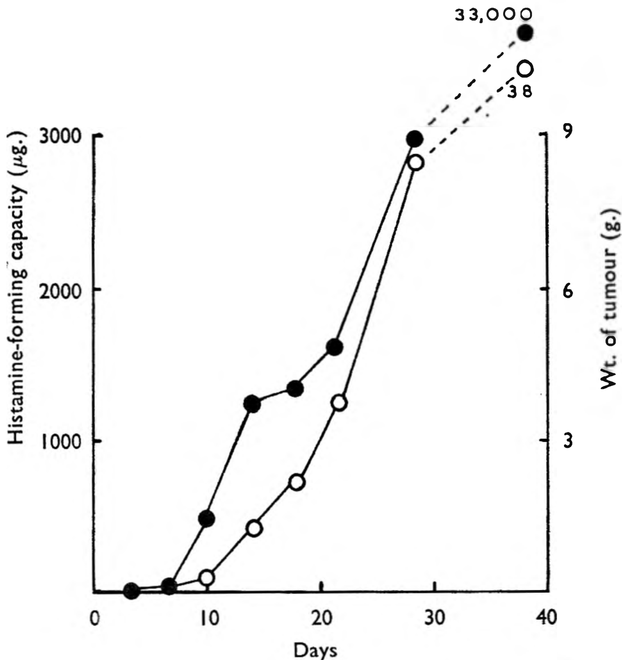

FIG. 1. Effect of age on the histamine-forming capacity (●——●) and weight (○——○) of the rat heptoma.

activity at an acid pH (6·5) and did not require the presence of an organic solvent, conditions which are similar to those already reported by Telford and West (1961b) for rat foetal liver. Both the tumour and the foetal liver had feeble 5-hydroxytryptamine-forming capacities when these were estimated by the method of Price and West (1960). These results are shown in Table I and compared with the corresponding properties of the enzyme in the adult liver. There is an inverse relation between the histidine decarboxylase and 5-hydroxytryptophan decarboxylase activities of these three tissues.

The histamine-forming capacity of the hepatoma steadily increased after grafting and high activities were found after 14 days. When portions of the tumour became necrotic (after about 20 days), activity increased still further. These results are shown in Fig. 1. Urinary excretion of histamine closely followed the changes in enzyme activity.

Similar results have been obtained after transplanting the same tumour into rats of the Wistar strain, although after grafting into several generations the histidine decarboxylase activity was reduced. Semicarbazide and α-methyl-DOPA in concentrations of 10^{-4} did not inhibit enzyme activity *in vitro*, and injections of cortisone also failed to inhibit tumour growth *in vivo*.

The results show that for this hepatoma there is an association between histamine-forming capacity and growth. However, little or no histidine decarboxylase activity has been found in other rapidly growing experimental tumours of rat and human origin. The rat hepatoma therefore seems to be a tumour arising from the foetal type of cell. Whether the function of its histidine decarboxylase is to produce histamine and so dilate blood vessels to increase the blood supply to the tumour tissue remains a question for future enquiry.

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