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RESEARCH PAPERS

THE ABSOLUTE CONFIGURATIONS OF THE α- AND β-METHYLCHOLINE ISOMERS AND THEIR ACETYL AND SUCCINYL ESTERS*

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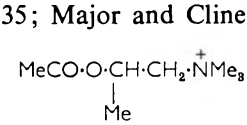
Received November 7, 1962

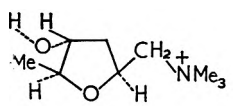
The absolute configurations of the (+)-acetyl-α- and -β-methylcholine iodides have been established, being related to D(−)-alanine hydrochloride and L(+)-lactic acid respectively. The (+)-, (−)- and racemic forms of the precursor amino-alcohols have been converted to the succinyl esters and these quaternised.

THE muscarinic activity of (+)-acetyl-β-methylcholine (I) (equiactive with acetylcholine) is about 100 times greater than that of the (−)-isomer (Major and Bonnett, 1935; Major and Cline, 1936).

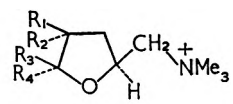


I

L(+)-Muscarine (II) (Hardegger and Lohse, 1957) is reported to be about 200 to 800 times more active than the D(−)-isomer (Gyermek and Unna, 1958).



II



III

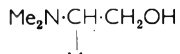
That the spatial arrangement of the substituents on the tetrahydrofuran ring of the muscarine isomers influences their activity is clearly shown by the fact that (±)-epi- (III; $R_1 = OH$, $R_2 = R_4 = H$, $R_3 = Me$), (±)-allo- (III; $R_1 = OH$, $R_2 = R_3 = H$, $R_4 = Me$) and (±)-epiallo-muscarine (III; $R_1 = R_3 = H$, $R_2 = OH$, $R_4 = Me$) possess only 1/300th, 1/150th and 1/100th respectively the muscarinic potency of (±)-muscarine (Gyermek and Unna, 1958; Waser, 1958).

It was of interest to establish the absolute configurations of the isomers of acetyl-α- and -β-methylcholine. This, it was considered, might allow a delineation of the stereochemical factors associated with possible receptor sites at which muscarinic activity is mediated.

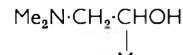
* See Beckett and others (1960 and 1961) for preliminary reports of some of the present work.

† One of the authors (J.W.C.) is indebted to the Medical Research Council for a Research Scholarship during the tenure of which this work, which forms a part of a Ph.D. thesis of the University of London, was carried out.

The (+)- and (−)-isomers of 2-dimethylaminopropan-1-ol (IV) and 1-dimethylaminopropan-2-ol (V) and also their racemic forms, prepared during this investigation, were converted to their succinyl esters and quaternised. These compounds were considered to be important in investigations into the stereochemical factors involved in neuromuscular blocking activity.

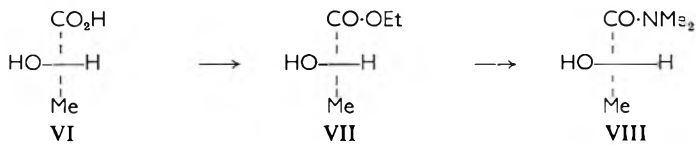


IV



V

(+)-Acetyl-β-methylcholine (XI) was related to L(+)-lactic acid(VI) by the following stereospecific route:

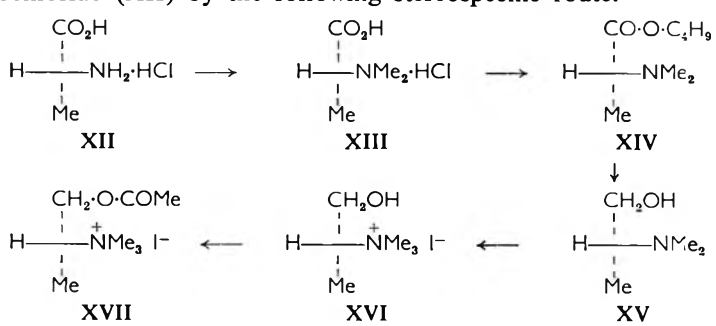


VI VII VIII

IX X XI

Zinc ammonium L(−)-lactate, prepared from lactic acid (VI), was converted to ethyl L(−)-lactate (VII), which on treatment with dimethylamine, followed by reduction with lithium aluminium hydride, gave L(+)-1-dimethylaminopropan-2-ol (IX), which on quaternisation with methyl iodide gave L(+)-1-dimethylaminopropan-2-ol methiodide (β-methylcholine iodide) (X). This on acetylation gave L(+)-1-dimethylaminoprop-2-yl acetate methiodide (acetyl-β-methylcholine iodide) (XI). Independently and at about the same time, Ellenbroek and van Rossum (1960) also established the absolute configuration of acetyl-β-methylcholine. The (+)- and (−)-isomers of 1-dimethylaminopropan-2-ol were prepared by resolution of the racemic alcohol using (+)-tartaric acid and α-bromo-(+)-camphor-π-sulphonic acid.

(+)-Acetyl-α-methylcholine (XVII) was related to D(−)-alanine hydrochloride (XII) by the following stereospecific route.

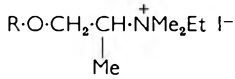


XII XIII XIV

XVII XVI XV

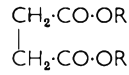
D(−)-Alanine hydrochloride (XII) was reductively methylated to give D(−)-2-dimethylaminopropionic acid hydrochloride (XIII) which on esterification with butanol gave butyl D(+)-2-dimethylaminopropionate (XIV). Reduction with lithium aluminium hydride gave D(−)-2-dimethylaminopropan-1-ol (XV), which on quaternisation with methyl iodide gave D(+)-2-dimethylaminopropan-1-ol methiodide (α-methylcholine iodide) (XVI). Acetylation with acetic anhydride gave D(+)-2-dimethylaminopropyl acetate methiodide (acetyl-α-methylcholine iodide) (XVII). L(−)-2-Dimethylaminopropyl acetate methiodide was prepared from L(+)-alanine hydrochloride by a similar synthetic route.

The (+)-, (−)- and (±)-forms of 2-dimethylaminopropan-1-ol were converted to the ethiodides (XVIII; R = H) and their acetyl derivatives (XVIII; R = MeCO).

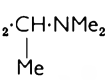


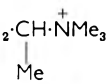
XVIII

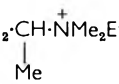
The optically active amino-alcohols were also used to prepare the succinyl esters and their quaternary derivatives of the general formula (XIX).

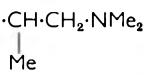


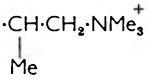
XIX

(a) R = (+)- and (−)- 

(b) R = (+)- and (−)- 

(c) R = (+)- and (−)- 

(d) R = (+)- and (−)- 

(e) R = (+)- and (−)- 

Preparation of the succinyl esters from the racemic alcohols would be expected to give a mixture of meso- and racemic forms. The presence of two distinct forms, which were considered to be the meso- and racemic forms, was established by vapour phase chromatography of the free bases. The quaternary derivatives of these esters [with the exception of compound (XIX c)] could not be separated by fractional crystallisation. The (±)-mixtures of 2-dimethylaminopropyl succinate methiodide,

1-dimethylaminoprop-2-yl succinate methiodide and 2-dimethylaminopropyl succinate ethiodide were prepared by mixing equal quantities of the optically active forms and crystallising from methanol-acetone.

An examination of the products of the interaction of the (+)- and (−)-1-dimethylaminopropan-2-ols with succinyl chloride by vapour phase chromatography indicated the presence of a mixture of two substances. On the basis of the gas chromatogram obtained with the succinate prepared from (±)-1-dimethylaminopropan-2-ol, these substances were considered to be the meso- and optically active forms. The appearance of two forms was unexpected but experiments have been carried out which have established that no racemisation or inversion of configuration of the optically active 1-dimethylaminopropan-2-ols occurred during the synthesis of their succinates. The apparent partial transformation of the optically active 1-dimethylaminoprop-2-yl succinates to the meso-form during gas chromatographic analysis is being investigated.

Experimental

Resolution of lactic acid. Lactic acid was resolved by a modification of the method of Purdie (1893) to give zinc ammonium L(−)-lactate $[\alpha]_D^{21}$ − 5·65° (*c* 8·3 in H_2O) [Purdie, 1893, quotes $[\alpha]_D$ − 6·06° (without stating concentration, solvent and temperature)].

Ethyl L(−)*-lactate.* Zinc ammonium L(−)-lactate (36 g.), $[\alpha]_D^{22}$ − 5·65° (*c* 8·3 in H_2O) was converted to lactic acid, which was then esterified with ethanol by a modification of the method Fischer and Mechel (1916) and gave ethyl L(−)-lactate (11·4 g.), b.p. 64–68° at 25 mm., $[\alpha]_D^{23}$ − 9·36° (*c* 3·16 in EtOH) [Freudenberg and Rhino, 1924, quote $[\alpha]_D^{20}$ − 10·3 to 10·5° (without stating concentration and solvent); Heilbron, 1953, quotes $[\alpha]_D^{14}$ − 10·33° (without stating concentration and solvent), b.p. 69–70° at 36 mm.].

L(+)-1-*Dimethylaminopropan-2-ol methiodide.* Ethyl L(−)-lactate (5 g.) and anhydrous dimethylamine (30 ml.) were heated in a sealed container for 1 hr. at 80° and then kept at room temperature for a further 24 hr. Excess of dimethylamine was removed by evaporation under reduced pressure and the residual oil dissolved in dry ether and reduced with lithium aluminium hydride (2 g.). After decomposition, the combined ethereal extracts were dried (anhyd. Na_2SO_4) and the ether removed by evaporation under reduced pressure. The residual liquid was distilled to give a basic fraction which contained optically active 1-dimethylaminopropan-2-ol, the specific rotation of which, based on the equivalent-weight determination, was $[\alpha]_D^{25}$ + 23·22° (*c* the equivalent of 0·9 pure L(+)-1-dimethylaminopropan-2-ol in EtOH). Treatment of an ethanolic solution with methyl iodide (2 ml.) in ethanol, followed by dropwise addition of ether, gave a solid which on crystallisation from ethanol-ether gave L(+)-1-dimethylaminopropan-2-ol methiodide m.p. 174–175°, $[\alpha]_D^{24.5}$ + 27·27° (*c* 2·2 in 90 per cent v/v EtOH) (Calc. for $C_6H_{16}INO$ equiv., 245. Found equiv., 246) [Major and Cline, 1936, quote $[\alpha]_D^{25}$ + 24·7° (without stating concentration and solvent) and m.p.

176–178° for (+)-1-dimethylaminopropan-2-ol methiodide prepared by resolution].

Resolution of 1-*dimethylaminopropan*-2-*ol.* (±)-1-Dimethylaminopropan-2-ol was resolved by the method of Major and others (1935; 1936) using (+)-tartaric acid and α-bromo-(+)-camphor-π-sulphonic acid and gave (−)-1-dimethylaminopropan-2-ol hydrogen (+)-tartrate $[\alpha]_D^{22.5}$ − 10·84° (*c* 4·9 in H_2O) [Major and Bonnett, 1935, quote $[\alpha]_D^{25}$ − 10·7° (without stating concentration and solvent)] and (+)-1-dimethylaminopropan-2-ol α-bromo-(+)-camphor-π-sulphonate, $[\alpha]_D^{24.5}$ + 80·6° (*c* 1·0 in H_2O) [Major and Bonnett, 1935, quote $[\alpha]_D$ + 83·5° (without stating solvent and temperature)].

L(+)-1-*Dimethylaminoprop*-2-*yl acetate methiodide.* L(+)-1-Dimethylaminopropan-2-ol methiodide (2 g.), m.p. 177–178°, $[\alpha]_D^{24}$ + 29·7° (*c* 2·0 in 90 per cent v/v EtOH) [obtained by quaternising L(+)-1-dimethylaminopropan-2-ol (prepared by resolution) with methyl iodide] was refluxed with acetic anhydride (25 ml.) for 30 min., excess of acetic anhydride removed by distillation under reduced pressure and the syrupy residue dissolved in methanol (10 ml.). Ether was added dropwise and the salt which formed was crystallised from methanol-ether to give L(+)-1-*dimethylaminoprop*-2-*yl acetate methiodide* (1·9 g.), m.p. 177·5–178·5°, $[\alpha]_D^{22.5}$ + 27·0° (*c* 2·0 in 90 per cent v/v EtOH) (Found: C, 33·6; H, 6·5; N, 5·0 per cent; equiv., 286. $C_8H_{18}INO_2$ requires C, 33·5; H, 6·3; N, 4·9 per cent; equiv., 287).

D(−)-1-*Dimethylaminopropan*-2-*ol methiodide.* To an ethereal solution of D(−)-1-dimethylaminopropan-2-ol [prepared from (−)-1-dimethylaminopropan-2-ol hydrogen (+)-tartrate (4 g.), $[\alpha]_D^{22.5}$ − 10·84° (*c* 4·9 in H_2O)] was added methyl iodide and ethanol. The solid which separated on standing was crystallised from ethanol-ether to give D(−)-1-dimethylaminopropan-2-ol methiodide (3·2 g.), m.p. 175·5–176·5°, $[\alpha]_D^{23}$ − 29·04° (*c*. 2·0 in 90 per cent v/v EtOH) (Calc. for $C_6H_{16}INO$ equiv., 245. Found: equiv., 244) [Major and Cline, 1936, quote m.p. 176·5–177·5°, $[\alpha]_D^{25}$ − 24·7° (without stating solvent and concentration)].

D(−)-1-*Dimethylaminoprop*-2-*yl acetate methiodide.* D(−)-1-Dimethylaminopropan-2-ol methiodide (2·5 g.) was acetylated in the manner described for L(+)-1-dimethylaminoprop-2-yl acetate methiodide and gave D(−)-1-*dimethylaminoprop*-2-*yl acetate methiodide* (2·6 g.) m.p. 176–178°, $[\alpha]_D^{23}$ − 27·38° (*c* 2·0 in 90 per cent v/v EtOH) (Found: C, 33·6; H, 6·1; N, 4·6 per cent; equiv., 286. $C_8H_{18}INO_2$ requires C, 33·5; H, 6·3; N, 4·9 per cent; equiv., 287).

Resolution of alanine. Alanine was resolved by a modification of the method of Pope and Gibson (1912) to give D(−)-alanine hydrochloride, $[\alpha]_D^{20}$ − 9·72° (*c* 13·1 in H_2O)* [Bowman and Stroud, 1950, quote $[\alpha]_D^{18}$ − 9·13° (*c* 13·1 in H_2O)] and L(+)-alanine hydrochloride, m.p. 200·5–201·5°, $[\alpha]_D^{20}$ + 9·47° (*c* 13·1 in H_2O) [Merck, 1960, quotes m.p. 204° and Harper, 1956, quotes $[\alpha]_D^{20}$ + 9·5° (*c* 13·1 in H_2O)].

* These figures are in accordance with those reported by Birnbaum and others (1952) who obtained $[\alpha]_D^{25}$ + 14·4° for L-alanine and $[\alpha]_D^{25}$ − 14·3° for D-alanine (*c* 4 in 5N HCl) by an enzymic method of resolution.

D(−)-2-*Dimethylaminopropionic acid hydrochloride.* D(−)-Alanine hydrochloride was reductively methylated by the method of Bowman and Stroud (1950) and gave D(−)-2-*dimethylaminopropionic acid hydrochloride*, m.p. 117·5–119°, $[\alpha]_D^{25\cdot5}$ − 14·47° (*c* 5·17 in H_2O) (Found: C, 38·3; H, 8·4 per cent; equiv., 154. $C_5H_{12}ClNO_2$ requires C, 39·1; H, 7·9 per cent; equiv., 154).

Butyl D(+)-2-*dimethylaminopropionate.* D(−)-2- Dimethylaminopropionic acid hydrochloride (27·1 g.) was esterified with butanol by a modification of the method of Fischer and Mechel (1916) to give *butyl* D(+)-2-*dimethylaminopropionate* (23·6 g.), b.p. 94–96° at 23 mm., $n_D^{18} = 1{\cdot}4264$, $[\alpha]_D^{24}$ + 25·1° (*c* 1·16 in EtOH) (Found: C,61·2; H, 11·0 per cent; equiv., 174. $C_9H_{19}NO_2$ requires C, 62·4; H, 11·1 per cent; equiv., 173).

D(+)-2-*Dimethylaminopropan*-1-*ol methiodide.* Butyl D(+)-2-dimethylaminopropionate (14 g.) in dry ether (75 ml.) was reduced with lithium aluminium hydride (3 g.) to give a basic oil having equiv. 138 (the calculated equivalent weight of D(−)-2-dimethylaminopropan-1-ol is 103). This oil consisted of a mixture of D(−)-2-dimethylaminopropan-1-ol and the butanol which co-distilled with the base. Correcting for the impurity, the amino-alcohol had $[\alpha]_D^{23\cdot5}$ − 3·83° (*c* the equivalent of 2·9 pure D(−)-2-dimethylaminopropan-1-ol in EtOH) [Mitchard (personal communication), found $[\alpha]_D^{23}$ − 2·8° (*c* 3·1 in EtOH), $n_D^{20} = 1{\cdot}4373$ (Calc. for $C_5H_{13}NO$ equiv., 103. Found: equiv., 107)]. A solution of the impure amino-alcohol (0·3 g.) was treated with methyl iodide (1 ml.) and the solid which separated crystallised from ethanol-ether to give D(+)-2-*dimethylaminopropan*-1-*ol methiodide* (0·6 g.), m.p. 298·5–299·5° (decomp.), $[\alpha]_D^{22\cdot5}$ + 4·15° (*c* 2·5 in 90 per cent v/v EtOH) (Found: C, 29·6; H, 6·6; N, 6·4 per cent; equiv., 242. $C_6H_{16}INO$ requires C, 29·4; H, 6·6; N, 5·7 per cent; equiv., 245).

D(+)-2-*Dimethylaminopropyl acetate methiodide.* D(+)-2-Dimethylaminopropan-1-ol methiodide (1 g.) was refluxed for 30 min. with acetic anhydride (25 ml.), the excess of which was removed by distillation under reduced pressure. The syrupy residue was crystallised from ethanol-ether to give D(+)-2-*dimethylaminopropyl acetate methiodide* (1g.), m.p. 107–108°, $[\alpha]_D^{20\cdot7}$ + 8·61° (*c* 5·0 in 90 per cent v/v EtOH) (Found: C, 33·2; H, 6·1; N, 5·6 per cent; equiv., 287. $C_3H_{18}INO_2$ requires C, 33·5; H, 6·3; N, 4·9 per cent; equiv., 287).

By similar procedures, L(+)-alanine hydrochloride, m.p. 200·5–201·5°, $[\alpha]_D^{20}$ + 9·47° (*c* 13·1 in H_2O)* was converted to L(+)-2-*dimethylaminopropionic acid hydrochloride*, m.p. 118·5–119·5°, $[\alpha]_D^{23\cdot4}$ + 14·3° (*c* 5·1 in H_2O) (Found: C, 38·0; H, 8·0; N, 8·9 per cent; equiv., 151. $C_5H_{12}ClNO$ requires C, 39·1; H, 7·9; N, 9·1 per cent; equiv., 154), which on esterification gave *butyl* L(−)-2-*dimethylaminopropionate*, b.p. 91–92° at 19 mm., $n_D^{22} = 1{\cdot}4241$, $[\alpha]_D^{26}$ − 26·6° (*c* 1·1 in EtOH) (Found: C, 63·1; H, 11·6; N, 8·1 per cent; equiv., 173. $C_9H_{19}NO_2$ requires C, 62·4; H, 11·1; N, 8·1 per cent; equiv., 173). This ester on reduction gave impure L(+)-2-dimethylaminopropan-1-ol, the specific rotation of the

* *Ibid*, p. 353.

pure alcohol, on the basis of the equivalent weight, being $[\alpha]_D^{24.5}$ — 2·26° (*c* the equivalent of 2·8 pure L(+)-2-dimethylaminopropan-1-ol in EtOH) [Mitchard (personal communication), found $[\alpha]_D^{23}$ + 2·4° (*c* 3·1 in EtOH), n_D^{20} = 1·4373 (Calc. for $C_5H_{13}NO$ equiv., 103. Found: equiv., 106)] and treatment of this with methyl iodide gave L(−)-2-*dimethylaminopropan*-1-*ol methiodide*, m.p. 299° (decomp.), $[\alpha]_D^{24.7}$ — 4·14° (*c* 2·5 in 90 per cent v/v EtOH) (Found: C, 30·0; H, 6·5; N, 5·7 per cent; equiv., 245. $C_6H_{16}INO$ requires C, 29·4; H, 6·6; N, 5·7 per cent; equiv., 245). Acetylation of the quaternary compound gave L(−)-2-*dimethylaminopropyl acetate methiodide*, m.p. 108–109°, $[\alpha]_D^{27}$ — 9·07° (*c* 5·0 in 90 per cent v/v EtOH) (Found: C, 33·5; H, 6·1 per cent; equiv., 289. $C_8H_{18}INO_2$ requires C, 33·5; H, 6·3 per cent; equiv., 287).

(±)-1-*Dimethylaminopropan*-2-*ol methiodide.* This was prepared by the addition of methyl iodide to a solution of (±)-1-dimethylaminopropan-2-ol (3 g.) in ethanol (20 ml.). The solid which separated was crystallised from ethanol-ether and gave (±)-1-dimethylaminopropan-2-ol methiodide (6·1 g.), m.p. 158·5–159·5° (Calc. for $C_6H_{16}INO$ equiv., 245. Found: equiv., 245).

(±)-1-*Dimethylaminoprop*-2-*yl acetate methiodide.* (±)-1-Dimethylaminopropan-2-ol methiodide (3 g.) was acetylated in the manner described for L(+)-1-dimethylaminoprop-2-yl acetate methiodide and gave (±)-1-dimethylaminoprop-2-yl acetate methiodide (3·2 g.), m.p. 137·5–138·5° (Calc. for $C_8H_{18}INO_2$ equiv., 287. Found: equiv., 286) (Merck, 1960, quotes m.p. 138–139·5°).

Ethyl (±)-2-*dimethylaminopropionate.* This was prepared by a modification of the method of Karrer (1922). A solution of dimethylamine (33 per cent in EtOH) (75 g.) was added to a solution of ethyl 2-bromopropionate (50 g.) in dry ether (150 ml.) and the mixture allowed to stand overnight. The solid which separated was removed and the filtrate diluted with ether (300 ml.) and allowed to stand overnight. The ethereal liquid was washed with a solution of sodium hydroxide (6 per cent) (2 × 6 ml.), dried (anhyd. Na_2SO_4) and evaporated under reduced pressure. The residual liquid was distilled, the fraction b.p. 74–76° at 40–42 mm. (23 g.) being collected (Calc. for $C_7H_{15}NO_2$ equiv., 145. Found: equiv., 165). The high equivalent weight obtained was due to non-basic contaminant (ethanol) and the presence of the required ester was confirmed by the preparation of the *picrate*, m.p. 83° (from ethanol) (Found: C, 41·4; H, 4·7; N, 15·6 per cent; equiv., 372. $C_{13}H_{18}N_4O_9$ requires C, 41·7; H, 4·8; N, 14·9 per cent; equiv., 374).

(±)-2-*Dimethylaminopropan*-1-*ol.* A solution of the impure ethyl (±)-2-dimethylaminopropionate (100 g.) in dry ether (145 ml.) was reduced with lithium aluminium hydride (26 g.). After reduction, the combined ethereal extracts were dried (anhyd. Na_2SO_4), the ether was removed by evaporation under reduced pressure and the residual liquid distilled to give a basic fraction (33·5 g.), b.p. 54–58° at 29 mm. (Calc. for $C_5H_{13}NO$ equiv., 103. Found: equiv., 110) which was considered to consist predominantly of (±)-2-dimethylaminopropan-1-ol and a small amount of ethanol. This fraction could not be further purified

and the amino-alcohol was identified by the picrate (from ethanol) m.p. 185° [Harper (personal communication), found m.p. 185°] (Calc. for $C_{11}H_{16}N_4O_8$ equiv., 332. Found: equiv., 328) and the methiodide.

(±)-2-*Dimethylaminopropan*-1-*ol methiodide.* A solution of (±)-2-dimethylaminopropan-1-ol (3 g.) in ethanol (5 ml.) was treated with methyl iodide (4·3 g.), the mixture allowed to stand for 20 min. and then ether added dropwise. The solid which separated was crystallised from ethanol-ether to give (±)-2-dimethylaminopropan-1-ol methiodide (7·0 g.), m.p. 299–300° (decomp.) (Calc. for $C_6H_{16}INO$ equiv., 245. Found: equiv., 242) (Karrer, 1922, quotes m.p. 296°).

(±)-2-*Dimethylaminopropyl acetate methiodide.* (±)-2-Dimethylaminopropan-1-ol methiodide (1 g.) was acetylated in the manner described for L(+)-1-dimethylaminoprop-2-yl acetate methiodide and gave (±)-2-*dimethylaminopropyl acetate methiodide* (0·8 g.), m.p. 131–132° (from ethanol-ether) (Found: C, 33·6; H, 6·2; N, 4·6 per cent; equiv., 286. $C_8H_{18}INO_2$ requires C, 33·5; H, 6·3; N, 4·9 per cent; equiv., 287).

(±)-2-*Dimethylaminopropan*-1-*ol ethiodide.* (±)-2-Dimethylaminopropan-1-ol (2 g.) in ethanol (10 ml.) was mixed with ethyl iodide (3 g.), heated to 60° and kept at room temperature overnight. Excess of ethanol was removed by evaporation and the solid which formed was crystallised from ethanol-ether to give (±)-2-*dimethylaminopropan*-1-*ol ethiodide* (1·5 g.), m.p. 285–285·5° (decomp.) (Found: C, 32·8; H, 7·1; N, 5·4 per cent; equiv., 259. $C_7H_{18}INO$ requires C, 32·5; H, 7·0; N, 5·4 per cent; equiv., 259).

(±)-2-*Dimethylaminopropyl acetate ethiodide.* (±)-2-Dimethylaminopropan-1-ol ethiodide (3 g.) was heated with acetic anhydride (25 ml.) for 30 min. at 70–80°. Excess of acetic anhydride was removed by distillation under reduced pressure and the syrupy liquid dissolved in ethanol (5 ml.). Ether was added dropwise and the salt which formed was crystallised from ethanol-ether to give (±)-2-*dimethylaminopropyl acetate ethiodide* (0·9 g.), m.p. 88° (Found: C, 35·9; H, 7·0; N, 4·5 per cent; equiv., 299. $C_9H_{20}INO_2$ requires C, 35·9, H, 6·7; N, 4·7 per cent; equiv., 301).

L(−)-2-*Dimethylaminopropan*-1-*ol ethiodide.* A solution of L(+)-2-dimethylaminopropan-1-ol in ethanol (3 ml.) was treated with ethyl iodide (3g.) in the manner described for (±)-2-dimethylaminopropan-1-ol ethiodide and gave L(−)-2-*dimethylaminopropan*-1-*ol ethiodide* (1·8 g.), m.p. 279·5–280·5° (decomp.), $[\alpha]_D^{21\cdot3}$ − 2·88° (*c* 2·5 in 90 per cent v/v EtOH) (Found: C, 33·3; H, 7·1; N, 5·3 per cent; equiv., 259. $C_7H_{18}INO$ requires C, 32·5; H, 7·0; N, 5·4 per cent; equiv., 259).

L(−)-2-*Dimethylaminopropyl acetate ethiodide.* L(−)-2-Dimethylaminopropan-1-ol ethiodide (1·8 g.) was acetylated in the manner described for (±)-2-dimethylaminopropyl acetate ethiodide and gave L(−)-2-*dimethylaminopropyl acetate ethiodide* (1·8 g.), m.p. 92–93°, $[\alpha]_D^{21\cdot5}$ − 11·27° (*c* 5·0 in EtOH) (Found: C, 36·3; H, 6·7; N, 4·6 per cent; equiv., 300. $C_9H_{20}INO_2$ requires C, 35·9; H, 6·7; N, 4·6 per cent; equiv., 301).

D(+)-2-*Dimethylaminopropan*-1-*ol ethiodide.* A solution of D(−)-2-dimethylaminopropan-1-ol in ethanol (3 ml.) was treated with ethyl iodide

(4 g.) by the method described for (±)-2-dimethylaminopropan-1-ol ethiodide and gave D(+)-2-*dimethylaminopropan*-1-*ol ethiodide* (3·5 g.), m.p. 272–273° (decomp. commences at about 250°), $[\alpha]_D^{22\cdot6}$ + 2·83° (*c* 2·5 in 90 per cent v/v EtOH) (Found: C, 32·2; H, 7·1; N, 5·4 per cent; equiv., 259. $C_7H_{18}INO$ requires C, 32·5; H, 7·0; N, 5·4 per cent; equiv., 259).

D(+)-2-*Dimethylaminopropyl acetate ethiodide.* D(+)-2-Dimethylaminopropan-1-ol ethiodide (1·9 g.) was acetylated by the method described for (±)-2-dimethylaminopropyl acetate ethiodide and gave D(+)-2-*dimethylaminopropyl acetate ethiodide* (2·0 g.), m.p. 90–91°, $[\alpha]_D^{25\cdot6}$ + 11·39° (*c* 5·0 in EtOH) (Found: C, 35·6; H, 7·0; N, 4·4 per cent; equiv., 303. $C_9H_{20}INO_2$ requires C, 35·9; H, 6·7; N, 4·7 per cent; equiv., 301).

L(−)-2-*Dimethylaminopropyl succinate methiodide.* L(+)-2-Dimethylaminopropan-1-ol (3·3 g.) was converted to the hydrochloride and heated with succinyl chloride (2·6 g.) in chloroform saturated with dry hydrogen chloride (20 ml.) at 90–100° for 6 hr. The chloroform was removed by distillation under reduced pressure and excess of succinyl chloride decomposed with iced water (1 ml.). The residue was made strongly alkaline with saturated sodium hydroxide solution and excess of anhydrous sodium carbonate added. The mass was extracted with ether (5 × 100 ml.) and the combined ethereal extracts dried (anhyd. Na_2SO_4). The ether was removed by evaporation under reduced pressure and the residual liquid distilled to give a basic fraction (3·5 g.), b.p. 108–146° at 0·3–0·7 mm., $n_D^{23\cdot5} = 1{\cdot}4508$, which contained optically active 2-dimethylaminopropyl succinate (Calc. for $C_{14}H_{28}N_2O_4$ equiv., 144. Found: equiv., 157). The specific rotation based on the equivalent weight was $[\alpha]_D^{25}$ − 3·35° (*c* the equivalent of 5·1 pure L(−)-2-dimethylaminopropyl succinate in EtOH). A solution of this liquid (3·2 g.) in acetone (20 ml.) was treated with methyl iodide (4 ml.) and the solid obtained crystallised from methanol-acetone to give L(−)-2-*dimethylaminopropyl succinate methiodide* (3·9 g.), m.p. 201·5–202·5° (decomp.), $[\alpha]_D^{27\cdot6}$ − 7·32° (*c* 5·0 in MeOH) (Found: C, 33·2; H, 6·1; N, 4·9 per cent; equiv., 287. $C_{16}H_{34}I_2N_2O_4$ requires C, 33·6; H, 6·0; N, 4·9 per cent; equiv., 286).

L(−)-2-*Dimethylaminopropyl succinate ethiodide.* A solution of L(−)-2-dimethylaminopropyl succinate (2·9 g.) in ethanol (3 ml.) was treated with ethyl iodide (4 g.) and the mixture kept overnight at room temperature. The solid which separated was crystallised from methanol-acetone to give L(−)-2-*dimethylaminopropyl succinate ethiodide* (2·3 g.), m.p. 159·5–160·5°, $[\alpha]_D^{23\cdot5}$ − 10·08° (*c* 5·0 in MeOH) (Found: C, 36·4; H, 6·5; N, 4·8 per cent; equiv., 298. $C_{18}H_{38}I_2N_2O_4$ requires C, 36·0; H, 6·4; N, 4·7 per cent; equiv., 300).

D(+)-2-*Dimethylaminopropyl succinate methiodide.* D(−)-2-Dimethylaminopropan-1-ol (3 g.) was converted to D(+)-2-dimethylaminopropyl succinate by the method described for L(−)-2-dimethylaminopropyl succinate methiodide. The distillate (2·3 g.) was contaminated with non-basic material and had an equivalent weight of 194. The equivalent

weight of D(+)-2-dimethylaminopropyl succinate is 144 and on the basis of this the specific rotation of the D(+)-2-dimethylaminopropyl succinate obtained was $[\alpha]_D^{25}$ + 3·42° (*c* the equivalent of 5·1 pure D(+)-2-dimethylaminopropyl succinate in EtOH). A solution of this (2·2 g.) in acetone (20 ml.) was treated with methyl iodide (2 ml.) and the solid obtained was crystallised from methanol-acetone to give D(+)-2-*dimethylaminopropyl succinate methiodide* (2·3 g.), m.p. 200·5–201° (decomp.), $[\alpha]_D^{24.5}$ + 7·23° (*c* 5·0 in MeOH) (Found: C, 33·9; H, 6·0; N, 5·2 per cent; equiv., 285. $C_{16}H_{34}I_2N_2O_4$ requires C, 33·6; H, 6·0; N, 4·9 per cent; equiv., 286).

D(+)-2-*Dimethylaminopropyl succinate ethiodide.* D(+)-2-Dimethylaminopropyl succinate (2·8 g.) was treated with ethyl iodide (4·0 g.) and gave D(+)-2-*dimethylaminopropyl succinate ethiodide* (2·8 g.), m.p. 159·5–160·5°, $[\alpha]_D^{24.5}$ + 9·6° (*c* 5·0 in MeOH) (Found: C, 36·2; H, 6·5; N, 4·5 per cent; equiv., 300. $C_{18}H_{38}I_2N_2O_4$ requires C, 36·0; H 6·4; N, 4·7 per cent; equiv., 300).

2-*Dimethylaminopropyl succinate methiodide.* (±)-2-Dimethylaminopropan-1-ol (3 g.) was converted to the hydrochloride and esterified with succinyl chloride by the method described for L(−)-2-dimethylaminopropyl succinate methiodide. A basic liquid (2 g.) was obtained on distillation, b.p. 134–138° at 0·28 mm. (Calc. for $C_{14}H_{23}N_2O_4$ equiv., 144. Found: equiv., 160), consisting mainly of 2-dimethylaminopropyl succinate, identified by the *perchlorate*, m.p. 126·5–128·5° (from acetic acid) (Found: C, 33·7; H, 6·0; N, 6·0 per cent. $C_{14}H_{30}Cl_2N_2O_{12}$ requires C, 34·4; H, 6·2; N, 5·7 per cent). It appeared likely that the basic material was a mixture of *meso-* and racemic forms. This was established by a gas chromatographic analysis (Column: silicone fluid and Daltolac; length, 6 ft.; temperature, 215°; katharometer temperature, 154°; bridge current, 130 mA; carrier gas, N_2; flow rate, 2·2 litres/hr.; pressure: inlet, 496 mm. Hg; outlet, 70 mm. Hg). The chromatogram showed two separate, well-defined peaks of different magnitude indicating the presence of distinct compounds in unequal proportions. Quaternisation of the basic liquid (1·9 g.) in acetone (10 ml.) with methyl iodide (2ml.) gave 2-dimethylaminopropyl succinate methiodide (2·7 g.), m.p. 212–213° (decomp.) (Calc. for $C_{16}H_{34}I_2N_2O_4$ equiv., 286. Found: equiv., 284) (Rosnati, 1951, quotes m.p. 213–215°). This material was considered to consist of a mixture of the *meso-* and racemic forms of the compound which could not be separated by crystallisation.

2-*Dimethylaminopropyl succinate ethiodide.* A solution of 2-dimethylaminopropyl succinate (2 g.) in ethanol (3 ml.) was treated with ethyl iodide (4 g.) and allowed to stand at room temperature overnight. The solid which separated was crystallised from methanol-acetone to give a crystalline solid (2·5 g.), m.p. 176°. Further recrystallisation from methanol-acetone gave meso-2-*dimethylaminopropyl succinate ethiodide*, m.p. 189·5–190° (after 5 recrystallisations) (Found: C, 36·2; H, 6·4; N, 4·9 per cent; equiv., 299. $C_{18}H_{38}I_2N_2O_4$ requires C, 36·0; H, 6·4; N, 4·7 per cent; equiv., 300). From the mother liquor, a crystalline solid was obtained, m.p. 169·5–170° (Found: C, 36·2; H, 6·5; N, 4·6 per

cent; equiv., 300. $C_{18}H_{38}I_2N_2O_4$ requires C, 36·0; H, 6·4; N, 4·7 per cent; equiv., 300) and was assumed to consist essentially of racemic 2-dimethylaminopropyl succinate ethiodide.

1-*Dimethylaminoprop-2-yl succinate methiodide.* (±)-1-Dimethylaminopropan-2-ol (3 g.) was converted to the hydrochloride and esterified with succinyl chloride by the method described for L(−)-2-dimethylaminopropyl succinate methiodide. A basic liquid (2 g.), b.p. 145–185° at 1·3–1·5 mm., containing 1-dimethylaminoprop-2-yl succinate as *meso*- and racemic forms was obtained. This liquid was subjected to gas-chromatographic analysis (Column: silicone elastomer 10 per cent on alkali-treated Celite; length, 6 ft.; temperature, 165°; bridge current, 130 mA; carrier gas, N_2; flow rate, 2 litres/hr.; pressure: inlet, 518 mm. Hg; outlet, 24 mm. Hg). The chromatogram showed two well-defined peaks (the first being the smaller; retention times, 7·0 min. and 37 min. respectively) indicating the presence of two forms of 1-dimethylaminoprop-2-yl succinate in a ratio of about 1 : 5·5 (preliminary experiments suggest that the *meso*-form is present in the greater amount). The basic liquid was quaternised with methyl iodide (2 g.) to give a solid which on crystallisation from methanol gave 1-dimethylaminoprop-2-yl succinate methiodide (2 g.), m.p. 234·5–235·5° (decomp.) (Calc. for $C_{16}H_{34}I_2N_2O_4$ equiv., 286. Found: equiv. 289) [Rosnati, 1951, quotes m.p. 235°; Vanderhaeghe and Derudder, 1952, quote m.p. 224–226° (decomp.)]. This compound was assumed to be a mixture of *meso*- and racemic forms, the former predominating.

D-(−)-1-*Dimethylaminoprop-2-yl succinate methiodide.* (−)-1-Dimethylaminopropan-2-ol hydrogen (+)-tartrate (9 g.), $[\alpha]_D^{22\cdot5}$ − 10·84° (*c* 4·9 in H_2O) was converted to (−)-1-dimethylaminopropan-2-ol hydrochloride and esterified with succinyl chloride by the method described for L(−)-2-dimethylaminopropyl succinate methiodide. A basic liquid (2·6 g.), b.p. 102–121° at 0·5–0·6 mm. (contaminated with non-basic material) was obtained (Calc. for $C_{14}H_{28}N_2O_4$ equiv., 144. Found: equiv., 175) having $[\alpha]_D^{24}$ − 12·5° (*c* the equivalent of 1·7 pure base in EtOH) calculated on the basis of the equivalent weight. A solution of the basic liquid (1 g.) in acetone (20 ml.) was treated with methyl iodide (2 ml.) and the solid which separated was crystallised from methanol-acetone to give *D*-(−)-1-*dimethylaminoprop-2-yl succinate methiodide* (1·6 g.), m.p. 246–247° (decomp.), $[\alpha]_D^{25}$ − 20·7° (*c* 2·0 in 90 per cent v/v MeOH) (Found: C, 33·7; H, 6·3; N, 4·9 per cent; equiv., 288. $C_{16}H_{34}I_2N_2O_4$ requires C, 33·6; H, 6·0; N, 4·9 per cent; equiv., 286).

L(+)-1-*Dimethylaminoprop-2-yl succinate methiodide.* (+)-1-Dimethylaminopropan-2-ol α-bromo-(+)-camphor-π-sulphonate (16·2 g.), $[\alpha]_D^{23}$ + 81·0° (*c* 1·0 in H_2O) was converted to (+)-1-dimethylaminopropan-2-ol hydrochloride and esterified with succinyl chloride. A basic liquid (1·7 g.) was obtained on distillation, b.p. 102–112° at 0·6–0·8 mm. (Calc. for $C_{14}H_{28}N_2O_4$ equiv., 144. Found equiv., 179), having $[\alpha]_D^{24}$ + 12·5° (*c* the equivalent of 1·75 pure base in EtOH) calculated on the basis of the equivalent weight. A solution of the basic liquid in acetone was treated with methyl iodide and the solid which separated

was crystallised from methanol-acetone to give L-(+)-1-*dimethylamino-prop-2-yl succinate methiodide* (1·8 g.), m.p. 246–247° (decomp.), $[\alpha]_D^{25}$ + 20·3° (*c* 2·0 in 90 per cent v/v MeOH) (Found: C, 33·8; H, 6·1; N, 4·7 per cent; equiv., 288. $C_{16}H_{34}I_2N_2O_4$ requires C, 33·6; H, 6·0; N, 4·9 per cent; equiv., 286).

Equal parts of the (+)- and (−)-forms of the quaternary succinyl esters were crystallised from methanol-acetone. In this way the following were prepared: (±)-1-dimethylaminoprop-2-yl succinate methiodide, m.p. 253·5–254·5° (decomp.); (±)-2-dimethylaminopropyl succinate methiodide, m.p. 195–196° (decomp.); (±)-2-dimethylaminopropyl succinate ethiodide, m.p. 160–161°.

Gas-chromatographic analysis of the succinates of the (±), (+) *and* (−)-1-*dimethylaminopropan-2-ols.* To ascertain which of the two peaks, observed in the gas chromatographic analysis of 1-dimethylaminoprop-2-yl succinate, was associated with the racemic form, equal quantities (0·2 g.) of the optically active forms were mixed and subjected to gas-chromatographic analysis (conditions as described under 1-dimethyl-aminoprop-2-yl succinate methiodide). Two peaks of approximately equal magnitude were obtained having retention times of 7 and 30 min. respectively. This suggested the possible contamination of the optically active forms with the meso-form. The (+)- and (−)-1-dimethylamino-prop-2-yl succinates were also subjected to gas chromatographic analysis under the same conditions and in each case two peaks of approximately equal magnitude were obtained, retention times being 8 and 38 min. and 8 and 32 min. respectively.

The origin of the meso-form in the optically active 1-*dimethylamino-prop-2-yl succinates.* The gas-chromatographic analyses suggested that the formation of the meso form arose either during the interaction of the optically active 1-dimethylaminopropan-2-ols with succinyl chloride or on the chromatographic column during the gas-chromatographic analysis. The following experiments served to establish that the formation of the meso-1-dimethylaminoprop-2-yl succinate did not take place during the preparation of the succinates from the optically active amino-alcohols:

1. A solution of (+)-1-dimethylaminopropan-2-ol α-bromo-(+)-camphor-π-sulphonate (4·5 g.), $[\alpha]_D^{23}$ + 80·1° (*c* 1·0 in H_2O) in water was made alkaline with a saturated solution of sodium hydroxide (10 ml.), excess anhydrous sodium carbonate added and the solid mass extracted with ether (5 × 100 ml.). The ethereal extracts were dried (anhyd. Na_2SO_4), treated with methyl iodide (5 ml.) and the solid which separated was crystallised from ethanol-ether to give L-(+)-1-dimethyl-aminopropan-2-ol methiodide (2·4 g.), m.p. 177–178°, $[\alpha]_D^{24}$ + 29·7° (*c* 2·0 in 90 per cent v/v EtOH) (Calc. for $C_6H_{16}INO$ equiv., 245. Found: equiv., 244).

2. An ethereal extract of (+)-1-dimethylaminopropan-2-ol, prepared from the α-bromo-(+)-camphor-π-sulphonate (1·2 g.), was shaken with dilute hydrochloric acid (2 × 10 ml.) and the aqueous layer evaporated to dryness under reduced pressure. The residue was dissolved in water

(1 ml.), the solution made alkaline with a saturated solution of sodium hydroxide (1 ml.), excess anhydrous sodium carbonate added and the solid mass extracted with ether (5 × 50 ml.). The ethereal extracts were dried (anhyd. Na_2SO_4), treated with a solution of methyl iodide (1 ml.) in ethanol (10 ml.) and the solid which separated crystallised from ethanol-ether to give L-(+)-1-dimethylaminopropan-2-ol methiodide (0·6 g.), m.p. 178–179°, $[\alpha]_D^{20}$ + 28·9° (*c* 2·0 in 90 per cent v/v EtOH) (Calc. for $C_6H_{16}INO$ equiv., 245. Found: equiv., 244).

3. A solution of (+)-1-dimethylaminoprop-2-yl succinate methiodide (0·6 g.), m.p. 246–247° (decomp.), $[\alpha]_D^{23}$ + 21·0° (*c* 2·0 in 90 per cent v/v MeOH) in water (40 ml.) was boiled under reflux for 1hr., cooled, and passed through a basic ion-exchange column (Amberlite IRA-400, OH). The basic eluate was evaporated under reduced pressure to about 3 ml. and acidified with a solution of hydriodic acid (55 per cent w/v) (1·5 ml.) in ethanol (30 ml.). The solution was evaporated to dryness and the solid crystallised from ethanol-ether to give L-(+)-1-dimethylaminopropan-2-ol methiodide (0·4 g.), m.p. 179–180°, $[\alpha]_D^{23}$ + 28·5° (*c* 2·0 in 90 per cent v/v EtOH) (Calc. for $C_6H_{16}INO$ equiv., 245. Found: equiv., 245).

4. To confirm that the optical activity of L-(+)-1-dimethylaminopropan-2-ol methiodide was unaffected by passage through the ion-exchange column, a solution of L-(+)-1-dimethylaminopropan-2-ol methiodide (0·6 g.), m.p. 178–179°, $[\alpha]_D^{20}$ + 28·9° (*c* 2·0 in 90 per cent v/v EtOH), in water (40 ml.) was passed through the ion-exchange column and the basic eluate worked up as previously described to give L-(+)-1-dimethylaminopropan-2-ol methiodide (0·5 g.), m.p. 178–179°, $[\alpha]_D^{22·5}$ + 28·5° (*c* 2·0 in 90 per cent v/v EtOH) (Calc. for $C_6H_{16}INO$ equiv., 245. Found: equiv., 246).

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THE IMPORTANCE OF STEREOISOMERISM IN MUSCARINIC ACTIVITY

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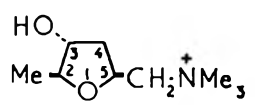
The muscarinic activities and rates of hydrolysis by acetylcholinesterase of the optical isomers of the acetyl-α- and acetyl-β-methylcholines of known configuration have been determined. The results have been correlated and the possible characteristics of muscarinic receptors outlined.

In a recent review, Waser (1961) has emphasised the importance of stereochemical factors in muscarinic activity. L(+)-Muscarine(I) (Hardeggar and Lohse, 1957) is 200 to 800 times more active than the D(−)-isomer. (±)-Epimuscarine, (±)-allomuscarine and (±)-epiallomuscarine, which are diastereoisomers of (±)-muscarine, have 1/300th, 1/150th and 1/100th respectively the activity of (±)-muscarine (Gyermek and Unna, 1958; Waser, 1958). On the other hand, (−)-muscarone(II) is only about twice as active as the racemic form and three times as active as its enantiomorph. The diastereoisomer of (±)-muscarone, (±)-allomuscarone(III) (Me and $CH_2 \cdot N^+Me_3$ *trans*), has about the same muscarinic potency as (+)-muscarone. The muscarinic activity of (+)-acetyl-β-methylcholine-(IV) was shown by Major and Bonnett (1935) and Major and Cline (1936)



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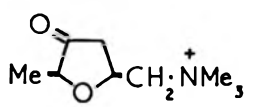

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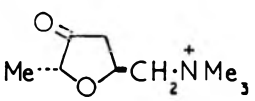

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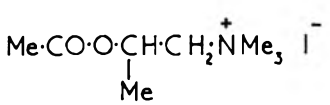

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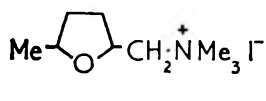

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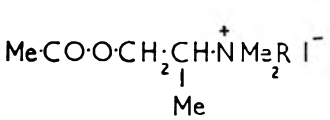


VI

to be about 100 times that of its enantiomorph. In muscarine, a *cis* arrangement of the 2–Me and 5–$CH_2 \cdot N^+Me_3$ groups with a 3–OH group in

* One of the authors (J. W. C.) is indebted to the Medical Research Council for a Research Scholarship during the tenure of which this work, which forms a part of a Ph.D. thesis of the University of London, was carried out.

a position *trans* to these appears to be necessary for muscarinic activity. Any variation in the arrangement or orientation of these three groups or the omission of one or more groups from the tetrahydrofuran ring markedly reduces activity. The importance of stereochemical factors in muscarinic activity in some but not all active molecules prompted us to investigate the muscarinic activities of 5-methyltetrahydrofurfuryltrimethylammonium iodide (V), the racemic and optically active forms of acetyl-α- (VI, R = Me) and acetyl-β-methylcholine(IV) iodides and 2-dimethylaminopropyl acetate ethiodide (VI, R=Et) with a view to providing further information about the stereochemical requirements for muscarinic activity.

Experimental Methods

Pharmacological

Guinea-pig ileum. Strips of ileum from freshly killed guinea-pigs were set up in Tyrode-Ringer at 32°. The spasmogenic activities of the test compounds were compared with that of acetylcholine chloride in 4-point assays of Latin square design. The spasmogenic action of these compounds was found to be unaffected by concentrations of hexamethonium bromide, 1×10^{-5}, and to be blocked by atropine sulphate, 1×10^{-8}.

Cat blood pressure. The vasodepressor activity of the test compounds was compared with that of acetylcholine chloride in 4-point assays of Latin square design, using cats anaesthetised with 80 mg./kg. chloralose. (Full experimental details will be published by E. Lesser.)

Enzymic Studies

Technique. All estimations were made using the Warburg manometric technique described by Ammon (1933) and modified by Augustinsson (1957) and Umbreit and others (1957).

Acetylcholinesterase source. This consisted of bovine erythrocyte stromata prepared by a modification of the method of Cohen and Warringa (1953) and Warringa and Cohen (1955). The freeze-dried enzyme preparation (approx. 75 mg.) was used to prepare an homogenate (100 ml.) in buffer solution consisting of NaCl, 0·117M; $NaHCO_3$, 0·034M; $MgCl_2 \cdot 6H_2O$, 0·001M buffered to pH 7·4 by the passage of a gas mixture, CO_2, 5 per cent; N_2, 95 per cent, for 20 min. and preserved with chloroform 0·1 per cent w/v.

Assay of esterase activity of acetylcholinesterase homogenates. The procedure used was essentially that described by Augustinsson (1957). Assays of esterase activity were done at 37° using acetylcholine iodide (AchI) at its optimum concentration ([S]opt) and 1·5 ml. of the homogenate, prepared as described, in a final volume of 3 ml. buffer solution. The concentration of enzyme in the homogenate was chosen such that the amount of CO_2 released during 30 min. under the conditions described was 80–130 μl. Homogenates having esterase activities of this order were used in all investigations made on the relevant esters.

Amount of acetylcholinesterase homogenate used. 1·5 ml. in all determinations.

Substrates. The following esters were used: acetylcholine iodide, (±)-, L-(+)- and D-(−)-acetyl-β-methylcholine iodide and (±)-, L-(−)- and D-(+)-acetyl-α-methylcholine iodide. The preparation of these has been described (Beckett, Harper and Clitherow, 1962).

Calculations. In the determinations of [S]opt. and the relative rates of hydrolysis of the relevant esters, the volume of CO_2 (μl.) released due to enzymic activity was plotted against time in min. The extrapolated 30 min. value used as the unit expressing cholinesterase activity was symbolised as $b_{30}^{1.5}$. Each manometric determination was always done in duplicate.

Measurement of optimum substrate concentrations. Estimations of the optimum substrate concentrations of the relevant esters were made at 37°. The concentration ([S]) of the substrates used in these determinations were within the range 1×10^{-3}M and $3{\cdot}3 \times 10^{-2}$M and the estimations were made using an acetylcholinesterase homogenate, previously assayed with AchI as described.

Substrate solutions. Solutions of a suitable strength were prepared in freshly prepared and gassed buffer solution immediately before use.

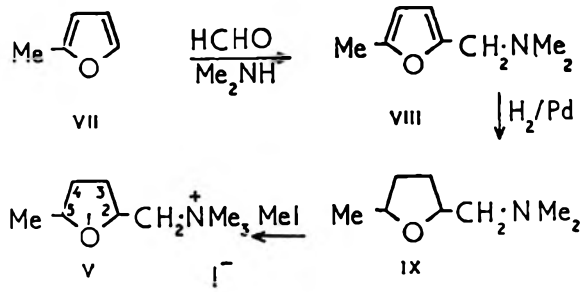
Determination of [S]opt. [S]opt. was determined for each substrate by plotting the value of $b_{30}^{1.5}$ against the corresponding $-\log[S]$ (or pS). At low substrate concentrations, $b_{30}^{1.5}$ values obtained by the extrapolation procedure were not accurate. More accurate results were obtained using a method of calculation involving the use of first order kinetics.

Absence of other esterases in the acetylcholinesterase preparation. The absence of the most likely other esterase contaminant (pseudocholinesterase) was established by measuring the degree of enzymic hydrolysis of acetyl-β-methylcholine (selective substrate for acetylcholinesterase, Augustinsson, 1957) in the absence and presence of the inhibitors Nu 1250 and Nu 683 (selective inhibitors of acetylcholinesterase and pseudocholinesterase respectively), inhibitor concentrations used being 10^{-7} and 10^{-8}M respectively (Hawkins and Gunter, 1946; Hawkins and Mendel, 1949).

Measurement of the relative rates of hydrolyis of the relevent compounds. The $b_{30}^{1.5}$ value obtained for each compound at its optimum concentration was compared with that of AchI using an assayed acetylcholinesterase homogenate. The rate of hydrolysis of each compound was calculated as a percentage of that of AchI (= 100 per cent). Several series of determinations were made using acetylcholinesterase homogenates of slightly differing strengths and the results were found to be in close agreement. The average percentage rates of hydrolysis of each compound are recorded in Table II (for full experimental details, see Clitherow, 1961).

Chemistry

5-Methyltetrahydrofurfuryltrimethylammonium iodide (V) was prepared by the following synthetic route:



5-*Methylfurfuryldimethylamine* (*VIII*). 2-Methylfuran was converted to 5-methylfurfuryldimethylamine by the method of Eliel and Peckham (1950). The free base had b.p. 85–86° at 49–53 mm. (calc. for $C_8H_{13}NO$ equiv., 139. Found: equiv., 144). The picrate (from ethanol) had m.p. 116·5–117·5°. (Calc. for $C_{14}H_{16}N_4O_7$ equiv., 368. Found: equiv., 366.) (Eliel and Peckham, 1950, quote m.p. 116–116·5°.)

5-*Methyltetrahydrofurfuryltrimethylammonium iodide* (*V*). 5-Methylfurfuryldimethylamine (13·5 g.) in acetic acid (140 ml.) was hydrogenated in the presence of palladium (10 per cent) on charcoal (6 g.). The solution was filtered, the acetic acid evaporated under reduced pressure and the residue made alkaline with a saturated solution of sodium hydroxide. Excess anhydrous Na_2CO_3 was added and the solid extracted with ether. The ethereal extracts were dried (anhyd. Na_2SO_4), the ether removed by evaporation under reduced pressure and the residual liquid distilled to give a fraction b.p. 86–89° at 54–55 mm. having an equivalent weight of 152. This basic fraction consisted mainly of 5-methyltetrahydrofurfuryldimethylamine and gave a *picrate* (from ethanol) m.p. 117·5–118·5°. (Found: C, 45·4; H, 5·6; N, 15·0 per cent; equiv., 373. $C_{14}H_{20}N_4O_8$ requires C, 45·2; H, 5·4; N, 15·1 per cent; equiv., 372.) Catalytic hydrogenation would be expected to give predominantly the *cis* isomer with probably a small proportion of the *trans* form (Eugster, 1960). A gas chromatogram (column, stationary phase: liquid paraffin and polyethylene glycol; inert support, Celite; column length, 6 ft.; column temperature, 94°; bridge current, 100 mA; carrier gas, N_2; flow rate, 2 litres/hr.; pressure: inlet 715 mm., outlet 285 mm. Hg) showed two closely aligned peaks indicating the presence of *cis* and *trans* isomers in the ratio of about 4 : 1.

A solution of the base (2 g.) in acetone (10 ml.) was treated with methyl iodide (2 ml.) at room temperature and the solid which separated was crystallised from ethanol-ether to give *cis-5-methyltetrahydrofurfuryltrimethylammonium iodide* (3 g.), m.p. 154–155°. (Found: C, 38·4; H, 7·1; N, 4·9 per cent; equiv., 285. $C_9H_{20}INO$ requires C, 37·5; H, 7·1; N, 4·9 per cent; equiv., 285.) The allocation of this configuration is based on the fact that the compound was isolated in high yield (80 per cent) and that the isomeric mixture had a well defined melting-point.

Acetylcholine analogues. The preparation of these has already been described (Beckett, Harper and Clitherow, 1962).

RESULTS

The muscarinic potencies of the relevant compounds are shown in Table I.

TABLE I

MUSCARINIC POTENCIES OF SOME ACETYLCHOLINE ANALOGUES AND THEIR OPTICAL ISOMERS (ACH = ACETYLCHOLINE)

Compound	Isomer	Guinea-pig ileum: Muscarinic activities. Mol. of drug = 1 mol. ACH. Mean limits P = 0·05		Ratio of activities of isomers (+)/(−)	Cat blood pressure: Muscarinic activities. Mol. of drug = 1 mol. ACH. Mean limits P = 0·05		Ratio of activities of isomers (−)/(−)
Acetyl-β-methylcholine iodide ..	(±)	1·58	1·35–1·91	240	0·97	0·76–1·22	280
	L-(+)-	1·01	0·94–1·10		0·71	0·54–0·93	
	D-(−)-	240	208–277		202	156–261	
Acetyl-α-methylcholine iodide ..	(±)	49	35–71	8	36	30–43	6
	L-(−)-	232	204–264		143	121–170	
	D-(+)-	28	22–35		25	21–29	
2-Dimethylaminopropyl acetate iodide	(±)	264	243–286	12	191	128–286	5
	L-(−)-	1,980	1,695–2,310		446	344–576	
	D-(+)-	170	156–186		91	69–114	
5-Methyltetrahydrofurfuryltrimethylammonium iodide	Mainly *cis*	20	18–21				

The rates of hydrolysis of acetylcholine and the racemic and optically active forms of the acetyl-α- and acetyl-β-methylcholine iodides at their optimum concentrations at 37° by acetylcholinesterase are shown in Table II.

TABLE II

RATES OF HYDROLYSIS OF (+), (−) AND (±) ACETYL-α- AND ACETYL-β-METHYLCHOLINE IODIDES COMPARED WITH ACETYLCHOLINE IODIDE (ACH) AT THEIR OPTIMUM CONCENTRATIONS AT 37° BY A STANDARD ACETYLCHOLINESTERASE HOMOGENATE

Compound	Isomer	[S]opt × 10^5M	Rates of hydrolysis compared with Ach (= 100 per cent)
Acetylcholine iodide		4·9	100
Acetyl-α-methylcholine iodide..	L-(−)-	6·7	97·4
	(±)	6·3	91·7
	D-(+)-	6·7	78·0
Acetyl-β-methylcholine iodide	L-(+)-	10·0	54·5
	(±)-	18·0	46·2
	D-(−)-	—	weak inhibition

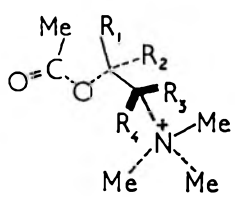
DISCUSSION

The intensity of muscarinic activity of cholinergically active compounds will, to some extent, be dependent upon their susceptibility to cholinesterase attack as well as their reaction with muscarinic receptors. Muscarine and muscarine-like compounds have been found to be weak inhibitors of acetylcholinesterase (Witkop, Durant and Friess, 1959)

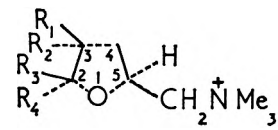
and their muscarinic activity may, therefore, be regarded as a measure of their interaction with a muscarinic receptor. The isomers of acetyl-α- and acetyl-β-methylcholine are, however, substrates or antagonists of acetylcholinesterase and susceptibility to cholinesterase attack must be taken into account when considering optimum structural requirements for fit at muscarinic receptors.

The absolute configuration of the (+)-isomers of acetyl-α-methylcholine (X; $R_1=R_2=R_3=H$, $R_4=Me$) (Beckett and others, 1961) and acetyl-β-methylcholine (X; $R_1=Me$, $R_2=R_3=R_4=H$) (Ellenbroek and van Rossum, 1960; Beckett, Harper, Clitherow and Lesser, 1961) have recently been determined and the configurational identity of L-(+)-acetyl-β-methylcholine with L-(+)-muscarine (XI; $R_1=R_4=H$, $R_2=OH$, $R_3=Me$) about C(5) established.

L-(+)-Muscarine is about 200–800 times more active than its enantiomorph and 2·5 times more potent than the (±)-form. The muscarinic activity is considerably reduced in those diastereoisomers of muscarine in which the Me, $CH_2{\cdot}N^+Me_3$ and OH groups are inverted, e.g. (±)-epi- (XI; $R_2=R_4=H$, $R_1=OH$, $R_3=Me$), (±)-allo- (XI; $R_2=R_3=H$, $R_1=OH$, $R_4=Me$) and (±)-epiallo-muscarine (XI; $R_1=R_3=H$, $R_2=OH$, $R_4=Me$) are about 1/300th, 1/150th and 1/100th respectively the activity



X

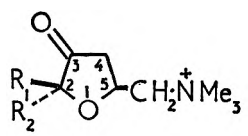


XI

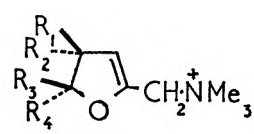
of (±)-muscarine. The muscarine type compounds have rigid structures in which the spatial arrangements of the substituent groups on the tetrahydrofuran ring are known. These compounds were used as models for a consideration of the most probable surface for presentation to a receptor. A diagramatic representation of the proposed muscarinic receptor has been made (Fig. 1) (Beckett and others, 1961) in which the quaternary N group and the ether oxygen, lying almost on a plane, form the two main centres for drug-receptor association while the –OH group is considered to act as a secondary site of association. In Fig. 1, the distance between the binding sites correspond approximately to the distances between the active centres in the muscarine molecule (Jellinek, 1957). The importance of the ether oxygen as a primary binding site is illustrated by the high muscarinic potencies of choline-ethyl ether and β-methylcholine-ethyl ether (Simonart, 1932). Molecular models would appear to indicate that if attachment of L-(+)-muscarine to the receptor takes place mainly by the quaternary nitrogen and ether oxygen, then one surface of the molecule, i.e. that which bears the *cis* arranged Me and $CH_2{\cdot}N^+Me_3$ groups, would not present a surface complementary to that of the receptor site.

It might be expected that due to steric factors, D-(−)-muscarine, (±)-2-methylmuscarine (XI; R_1=H, R_2=OH, R_3=R_4=Me) and the diastereoisomers of (±)-muscarine would not be able to present a surface complementary to that of the depicted receptor. Other analogues of muscarine (XI; R_1=H, R_2=OH, R_3 or R_4=Pr; and XI; R_1=OH, R_2=H, R_3 or R_4=Pr; and XI; R_1=H, R_2=OH, R_3 or R_4=Bu) also show reduced muscarinic activity and this may be attributed to steric factors associated with the propyl and butyl groups in the 2-position of the muscarine molecule. The importance of having one methyl group in the 2-position and a correctly orientated 3-OH group is exemplified by the relatively low activities of the *trans* and *cis* forms of (±)-desmethylmuscarine (*trans*; XI; R_2=OH, R_1=R_3=R_4=H) (*cis*; XI; R_1=OH, R_2=R_3=R_4=H) which have respectively about 1/100th and 1/1000th the muscarinic activity of acetylcholine. The importance of the –OH group and its orientation *trans* to the $CH_2{\cdot}N^+Me_3$ group is exemplified by the fact that (±)-tetrahydrofurfuryl trimethylammonium (XI; R_1=R_2=R_3=R_4=H) has only about 1/100th to 1/300th the activity of acetylcholine. A similar pattern of configurational dependence for activity is observed in the series-(±)-muscarine (XI; R_1=R_4=H, R_2=OH, R_3=Me), (±)-5-methyltetrahydrofurfuryltrimethylammonium (V) and (±)-epimuscarine (XI; R_2=R_4=H, R_1=OH, R_3=Me) which have about 4/5th, 1/20th and 1/300th the activity of acetylcholine respectively.

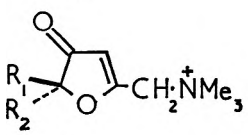
(±)-Muscarone (XII; R_1=Me, R_2=H) is about six times more potent than (±)-muscarine. This may be due to the greater polarity of the keto group which may facilitate binding to site 3 of the receptor and to the



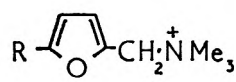
XII



XIII



XIV



XV

greater planarity of the molecule which may permit a closer drug-receptor association. The greater degree of planarity might explain why (−)-muscarone is only three times more active than its enantiomorph and about twice as active as the (±)-form, in distinct contrast to larger differences in activity between the less planar (+) and (−)-muscarine isomers. An anomaly appears to exist concerning the relationship between configuration and activity of the muscarone isomers. The more active (−)-muscarone is reported to have the D- configuration at C(5) (Waser, 1962), i.e. the inverse configuration to (+)-muscarine at this centre, but the chemical evidence is not unequivocal.

Neither isomer of (±)-allomuscarone (XII; R_1=H, R_2=Me) which has about 1/2 the activity of (±)-muscarone, would be expected to present a surface complementary to the depicted receptor. (±)-Allomuscarone is a more planar molecule than (±)-allo- and (±)-epiallomuscarine [from which it may be derived chemically (Eugster and others, 1958a, b, c)]. The increased planarity in (±)-allomuscarone would diminish the steric effect of the 2-methyl group and allow a closer drug-receptor association than in the case of the above mentioned precursors.

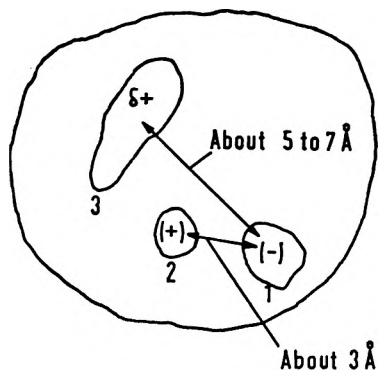


FIG. 1. Diagramatic representation of muscarinic receptors.

1. Anionic cavity negatively charged to accommodate quaternary nitrogen. 2. Positively charged point accommodating ether linkage of muscarine or ester linkage of acetylcholine and its analogues. 3. (+) Charged area to accommodate OH of muscarine, C = O of acetylcholine and its analogues or double bond of furan analogues of muscarine.

The importance of planarity in muscarinic activity is further exemplified by the high activities of (±)-4,5-dehydromuscarine (XIII; R_1=R_4=H, R_2=OH, R_3=Me), (±)-4,5-dehydro-epimuscarine (XIII; R_2=R_4=H, R_1=OH, R_3=Me), (±)-4,5-dehydromuscarone (XIV; R_1=Me, R_2=H) [which may exist in equilibrium with the enolic form (Waser, 1962)] and 5-methylfurfuryltrimethylammonium (XV; R=Me) which have respectively 1, 1/2, 5·3 and 1/3 to 1 times the activity of acetylcholine (Gyermek and Unna, 1958, 1960a, b; Ing, Kordik, Tudor-Williams, 1952).

The importance of the methyl group in the 2-position of muscarine (or the 5-position of furan analogues) and the steric implications of this group have been stressed. It is not unreasonable to suppose that this group contributes to an increased electron availability in the vicinity of the ether oxygen which would facilitate drug-receptor association, e.g. the muscarinic potencies of (±)-desmethylmuscarone (XII; R_1=R_2=H) and furfuryltrimethylammonium (XV; R=H) have respectively only about 1/40th and 1/10th the activity of their methyl analogues (Zwicky, Waser and Eugster, 1959; Armitage and Ing, 1954; Ing and others, 1952).

The determination of the absolute configurations of the acetyl-α- and acetyl-β-methylcholine isomers and the configurational identity of the

latter with L(+)-muscarine about C(5) permits consideration of the fit of these compounds at the proposed muscarinic receptor. It is assumed that acetylcholine and the acetyl-α- and acetyl-β-methylcholine isomers adopt conformations similar to that of L(+)-muscarine when acting at the muscarinic receptors. The (+)-isomer of acetyl-β-methylcholine (X; R_1=Me, R_2=R_3=R_4=H) can adopt a conformation complementary to that of the depicted receptor while the (−)-isomer (X; R_1=R_3=R_4=H, R_2=Me) cannot do so. Simonart (1932) showed that the muscarinic activity of (±)-acetyl-α-methylcholine was about 1/20th that of acetylcholine which suggests that the α-methyl group might exert a steric effect on the cationic head thereby preventing a close drug-receptor association. The muscarinic activity of D-(+)-acetyl-α-methylcholine (X; R_1=R_2=R_3=H, R_4=Me) is about 7 times greater than its enantiomorph (X; R_1=R_2=R_4=H, R_3=Me) (Table I). Both isomers of acetyl-α-methylcholine can probably present surfaces complementary to the receptor, the only difference between the two isomers, when in the desired conformation, being in the direction in which the methyl groups project. One would, therefore, expect these isomers to have not greatly dissimilar low potencies.

As indicated previously, the hydrolysis of the optical isomers of acetyl-α- and acetyl-β-methylcholine by acetylcholinesterase is a complicating factor in the interpretation of their relative muscarinic potencies. Since the muscarine-like molecules are not susceptible to cholinesterase hydrolysis and are only weak inhibitors of acetylcholinesterase, their muscarinic potencies may be interpreted as being a measure of their relative interactions at the muscarinic receptors.

The muscarinic potency of L(+)-acetyl-β-methylcholine is about the same as that of acetylcholine whereas its rate of hydrolysis by acetylcholinesterase is about 54 per cent that of acetylcholine (Table II). The slower rate of hydrolysis of L(+)-acetyl-β-methylcholine in the biophase of the muscarinic receptors probably compensates for the deleterious effect of a β-methyl group on the association of this molecule and a muscarinic receptor with the result that acetylcholine and L(+)-acetyl-β-methylcholine have equimuscarinic potencies.

The weak inhibitory action of D(−)-acetyl-β-methylcholine towards acetylcholinesterase (Table II) has been shown by Beckett and others (1961) to reinforce slightly the muscarinic activity of the L(+)-isomer in (±)-acetyl-β-methylcholine.

Acetylcholinesterase hydrolyses D(+)- and L(−)-acetyl-α-methylcholine at 78 per cent and 97 per cent of the rate of acetylcholine respectively (Table II). The L(−)-isomer probably presents a more favourable complementary conformation to the active site of acetylcholinesterase than does its enantiomorph. L(−)-Acetyl-α-methylcholine might also present a more favourable complementary conformation to the muscarinic receptors than the D(+)-isomer but, owing to the faster rate of enzymic hydrolysis and inactivation of the former isomer in the biophase of the muscarinic receptors, the latter isomer would be expected to exert the greater muscarinic activity.

Replacement of a methyl group in the cationic head of acetylcholine by an ethyl group causes a threefold reduction in muscarinic activity (Ing, 1949; Holton and Ing, 1949; Ing and others, 1952). A similar effect was observed in the present investigations in which the muscarinic potencies of D(+)-, L(−)- and (±)-2-dimethylaminopropyl acetate ethiodide were found to be about 1/5th of the corresponding acetyl-α-methylcholine isomers (Table I).

Since (±)-tetrahydrofurfuryltrimethylammonium (XI; $R_1=R_2=R_3=R_4=H$) and (±)-β-methylcholine ethyl-ether have about 1/100th to 1/300th and 1/20th to 1/40th the muscarinic activity of acetylcholine respectively (Simonart, 1932; Fellows and Livingston, 1940), the presence of the 2-Me and 3-OH is important in rigid muscarine-type molecules. Such groups do not appear to be essential if flexibility exists in the molecules, i.e. in acetylcholine-like molecules.

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ABSENCE OF CAPILLARY PERMEABILITY RESPONSE IN RATS TO DEXTRAN AND EGG-WHITE

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Intradermal dextran and ovomucoid fail to increase capillary permeability in rats resistant to intraperitoneal dextran although intradermal histamine, 5-HT and compound 48/80 are as effective as in control rats. When the skin of control rats is depleted of its histamine, intradermal dextran and ovomucoid are first ineffective but later increase capillary permeability although the skin histamine remains low. Chronic treatment of control rats with intraperitoneal dextran (which only slightly reduces both the skin histamine and 5-HT) prevents the local dextran and ovomucoid responses but does not affect those of compound 48/80, histamine and 5-HT. It is concluded that intradermal dextran and ovomucoid increase capillary permeability in rats by a mechanism involving substances other than histamine and 5-HT, and that this mechanism is absent in rats which do not respond to intraperitoneal dextran.

THE inflammatory anaphylactoid reaction produced in rats by the single intraperitoneal injection of dextran or egg-white has been shown to be mediated chiefly through a release of 5-hydroxytryptamine (5-HT) and histamine (Parratt and West, 1957). Recently, Harris and West (1961) found that not all rats react to this primary injection although both amines are always present in the skin of rats. We have studied this problem further by determining the intensity of colloid-dye accumulation in the abdominal skin of both types of rat after various treatments.

METHODS

Groups of Wistar albino rats obtained from the Agricultural Research Council's Field Station at Compton were used in all experiments. They were injected intraperitoneally with dextran (Intradex, 180 mg./kg.) and subsequently divided into two groups—those which showed pruritus and oedema of the face, tongue and paws (hereinafter called Reactors), and those which failed to show the anaphylactoid reaction (called Non-reactors). The animals initially resistant to dextran (about 25 per cent of the total number) were also resistant to doses of 30, 120 and 480 mg./kg. when tested at weekly intervals: they were also resistant to fresh egg-white (10 ml./kg.).

Tests for capillary permeability. The abdominal skin of the rats (body weight 150–200 g.) was depilated with an electric razor 24 hr. before the test. The animals were injected intravenously on the day of the test with azovan blue dye (7 mg./kg.), and then intradermally at the left and right sides of the midline of the shaved skin with the agents stated below in volumes of 0·1 ml. Thirty min. later, the rats were killed and the shaved skin was removed and firmly pinned to a cork board. The extent

of the colloid dye accumulation was estimated on the inner side of the skin by measuring the average diameter of the extent of blueing. Extent and intensity of blueing were usually of a similar order. The mean response to neutralised saline (NaCl, 0·9 per cent w/v) was 10 ± 1 mm. (100 determinations). In each experiment, groups of at least 3 reactor and 3 non-reactor rats were used, and the results averaged for each type.

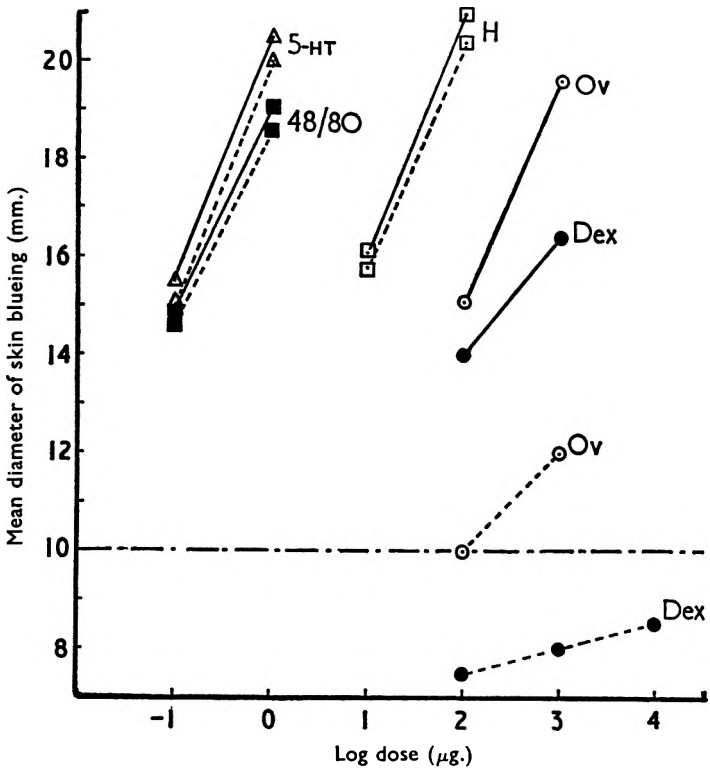

FIG. 1. Dye accumulation in the skin when 5-HT (△), compound 48/80 (■), histamine (□), ovomucoid (○), and dextran (●) are injected intradermally into reactor (continuous lines) and non-reactor (broken lines) rats. Abscissa, dose in μg. (log scale); ordinate, mean diameter of blueing in mm. Mean saline response is 10 ± 1 mm. (shown by dotted line).

Agents used. Six intradermal injections were always given to each rat. These were histamine (10 or 100 μg.), 5-HT (0·1 or 1 μg.), compound 48/80 (0·1 or 1 μg.), ovomucoid (100 or 1000 μg.), dextran (Intradex, approximate molecular weight 145,000, dose 100 or 1000 μg.) and saline (0·1 ml.). In some experiments, dextrans of two other molecular weights (4,000 and 20 million) were used each in doses of 100 or 1000 μg.

Depletion of amines. Polymyxin B was injected intraperitoneally to deplete rats of their skin histamine before the test; the twice daily doses were 2·5 mg./kg. on the first day, 5 mg./kg. on the second, and 7·5 mg./kg.

on the third. Tests of capillary permeability were carried out using the smaller doses of the five agents on the fourth, eighth and tenth days, that is 1, 5 and 7 days after the last dose of polymyxin. Compound 48/80 was injected intraperitoneally to deplete rats of their skin histamine and part of their skin 5-HT before the test; the twice daily doses were 1 mg./kg. on the first day, 2 mg./kg. on the second and 3 mg./kg. on the

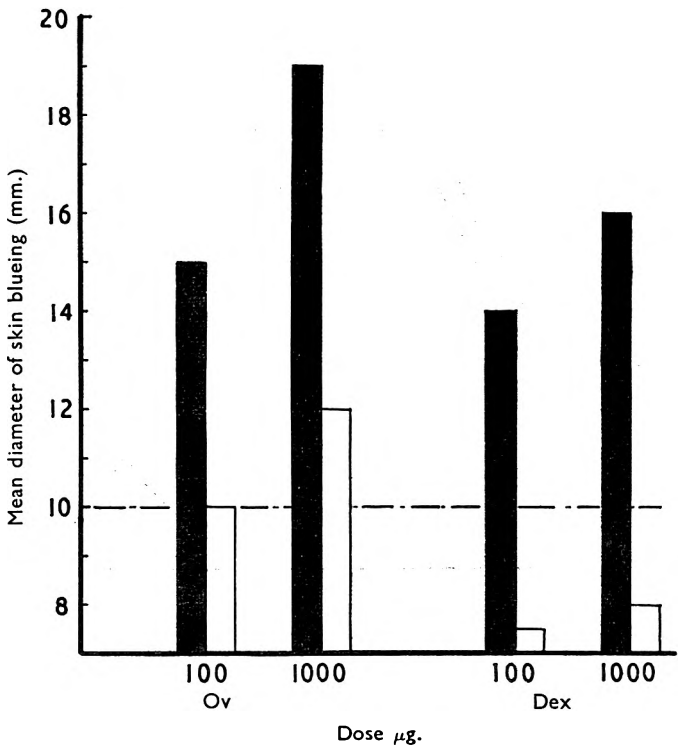

FIG. 2. Dye accumulation in the skin when ovomucoid and dextran (100 and 1,000 μg. doses) are injected intradermally in reactor (shaded columns) and non-reactor (plain columns) rats. Note that the lower dose of ovomucoid and both doses of dextran are ineffective in non-reactor rats.

third. Animals were then tested as previously described. Reserpine was injected intraperitoneally to deplete rats of their skin 5-HT before the test; the doses used were 1 mg./kg. daily for 3 days, the rats being subsequently used as described above.

In other experiments, gradually increasing doses of dextran were given daily to produce resistance to dextran in reactor rats; these intraperitoneal doses started at 600 mg./kg. and finished at 3,000 mg./kg. on the seventh day, the rats being tested on the eighth day.

Antagonists. These were given intravenously 30 min. before the azovan blue dye and active agent. UML 491 (1-methyl-lysergic acid butanolamide), a specific antagonist of 5-HT, was used in doses of 25–250

μg./kg. Mepyramine, a specific antagonist of histamine, was used in doses of 500–2,500 μg./kg. In a few experiments, both antagonists were used simultaneously.

Histamine content of the abdominal skin. This was estimated in control rats and in rats treated with polymyxin B, compound 48/80, reserpine and dextran. The method of Parratt and West (1957) was used.

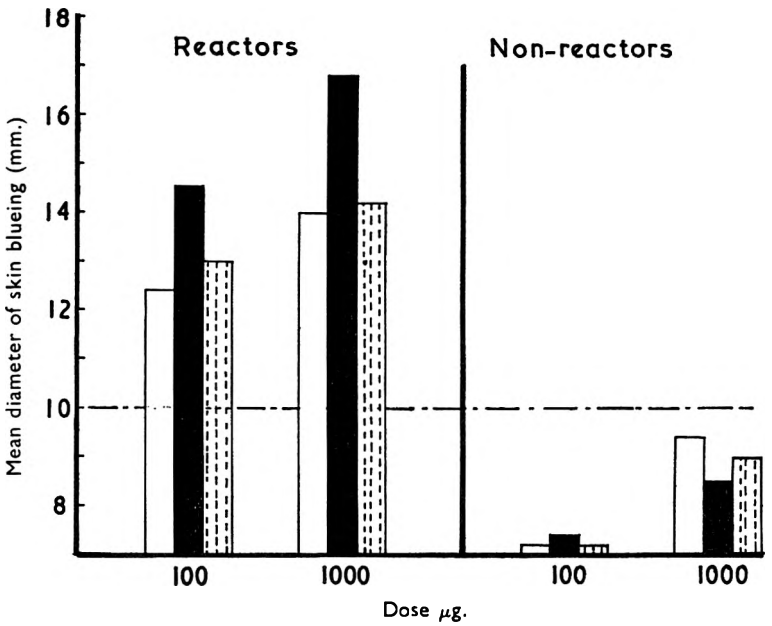

FIG. 3. The effect of intradermal injections of dextrans of different molecular weight. Dye accumulation only occurs in reactor rats. Open column, mol. wt. 4×10^3. Solid column, mol. wt. 145×10^3. Dotted column, mol. wt. 20×10^6.

RESULTS

The relation between dose and response. This is shown in Fig. 1 for the five agents. Compound 48/80 and 5-HT were the most potent and were equally effective in both reactor and non-reactor rats. Histamine was similarly effective in both types of animal but it was about 100 times less potent (Sparrow and Wilhelm, 1957). Ovomucoid and dextran were some 10 times less active than histamine in reactor rats but gave no response in non-reactors. Occasionally, ovomucoid but not dextran produced areas of blueing which exceeded those of saline. Higher doses of dextran, for example 10 mg., were also ineffective in non-reactor rats (see Fig. 1). A comparison of the responses of dextran and ovomucoid in reactor and non-reactor rats is more clearly shown in Fig. 2.

Effect of dextrans of different molecular weight. The three dextrans produced responses in reactor rats but all were ineffective in non-reactors. These results are recorded in Fig. 3. The most active sample had a molecular weight of about 145,000.

Effect of amine depletion. Chronic treatment of reactor rats with polymyxin B prevented the responses of compound 48/80, ovomucoid and dextran but only slightly reduced those of histamine and 5-HT when the test was carried out 1 day after treatment (see Fig. 4). The polymyxin treatment lowered the skin histamine by 81 per cent (control value, 25 μg./g.) and disrupted the mast cells. When tested 4 days later, the

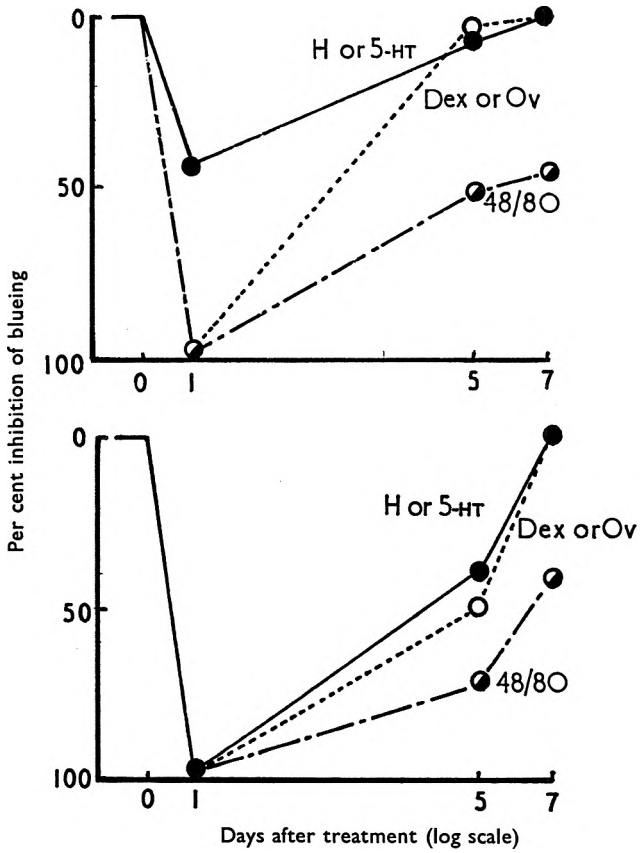

FIG. 4. Inhibition of the dye accumulation in the skin of rats by previous treatment with polymyxin B (upper tracing) or compound 48/80 (lower tracing). Values shown for histamine and 5-HT (●) have been averaged, also those of ovomucoid and dextran (○). Note that the compound 48/80 response (◕) is still reduced after 7 days of recovery.

responses to ovomucoid and dextran had fully recovered although the skin histamine remained low at 20 per cent of the control value; the response of compound 48/80 was still greatly reduced (see Table I). A similar result was obtained with histamine, 5-HT and compound 48/80 when the test was made in non-reactor rats.

Chronic treatment of reactor rats with compound 48/80 prevented the responses of compound 48/80, ovomucoid, dextran, histamine, and 5-HT

(see Fig. 4). This treatment lowered the skin histamine by 83 per cent. All the responses (except that of compound 48/80) had fully recovered when the test was carried out 7 days later, although the skin histamine was still depleted (see Table I). Again, a similar result was obtained with compound 48/80, histamine and 5-HT when the test was carried out in non-reactor rats.

Chronic treatment of reactor rats with reserpine prevented the responses of dextran and ovomucoid but only slightly reduced those of compound 48/80, histamine and 5-HT. However, chronic treatment of reactor rats with dextran, which only slightly reduced both the skin histamine and 5-HT, prevented the responses of dextran and ovomucoid but did not reduce those of compound 48/80, histamine and 5-HT; such reactor rats then are reacting like untreated non-reactor rats.

TABLE I

RESPONSES OF THE 5 AGENTS WHEN INJECTED INTRADERMALLY INTO REACTOR AND NON-REACTOR RATS, ON A RELATIVE SCALE FROM 0 TO ++. THE RESULTS WITH REACTOR RATS CHRONICALLY TREATED WITH DIFFERENT COMPOUNDS ARE ALSO SHOWN; (*a*) TESTS COMPLETED 1 DAY AFTER TREATMENT, AND (*b*) TESTS COMPLETED 7 DAYS LATER. THE SKIN HISTAMINE VALUES ARE GIVEN AS PERCENTAGES OF THE CONTROL VALUES (25 μG./G.)

Agent	Dose (μg.)	Reactor rats	Non-reactor rats	Chronic treatment of reactor rats					
				Dextran	Reserpine	Polymyxin B		Compound 48/80	
						(*a*)	(*b*)	(*a*)	(*b*)
Ovomucoid	100	++	0	0	0	0	++	0	++
Dextran	100	++	0	0	0	0	++	0	++
Histamine	10	++	++	++	++	±	++	0	++
Compound 48/80	0·1	++	++	++	++	0	±	0	±
5-HT	0·1	++	++	++	++	+	++	0	++
Skin histamine per cent		100	100	78	80	19	20	17	15

Effect of antagonists. UML 491 was ineffective at doses of 25 μg./kg. but it prevented the 5-HT response in both reactor and non-reactor rats when the dose was doubled. At this dose level, it also reduced the responses of dextran, ovomucoid and compound 48/80 in reactor rats but had no effect on the histamine response. Increasing the dose to 250 μg./g. prevented all responses except that of histamine.

Mepyramine in doses of 1 mg./kg. reduced the histamine response in both reactor and non-reactor rats but failed to alter the responses to 5-HT, compound 48/80, ovomucoid and dextran in reactor rats. Increasing the dose to 2·5 mg./kg. completely prevented the histamine reaction but still did not affect the other four responses.

When UML 491 (25 μg./kg.) and mepyramine (1 mg./kg.) were given together, the histamine response was reduced but the other four were prevented. The small dose of mepyramine potentiated the action of a small dose of UML 491 producing a result which is similar to that of a larger dose of UML 491.

Discussion

The results show that rats which are resistant to intraperitoneal doses of dextran (the non-reactors) are also resistant to intradermal dextran and ovomucoid although the reactions to intradermal compound 48/80, histamine and 5-HT are similar to those in reactor rats. Such a result suggests that non-reactors either possess an antagonist to dextran and ovomucoid or are deficient in one or more of the components necessary to effect the local capillary response. Non-reactor rats contain as much histamine and 5-HT as do reactors and so it may be that an intermediate stage in the reaction is unable to occur in non-reactor animals. Further, when reactor rats had received many doses of intraperitoneal dextran so that they failed to show a reaction to intraperitoneal or intradermal dextran or ovomucoid, they still reacted to compound 48/80, histamine and 5-HT.

Reactor rats treated chronically with intraperitoneal polymyxin B became refractory to intradermal compound 48/80, dextran and ovomucoid but not to histamine and 5-HT, whereas treatment with compound 48/80 completely prevented all reactions. In the days after treatment, the responses of dextran, ovomucoid, histamine and 5-HT were quickly regained, although the skin histamine was still at low levels, but that of compound 48/80 remained suppressed. Thus released histamine and 5-HT may respond in a different manner from exogenous histamine and 5-HT, and other factors are probably involved in the intradermal reaction, even in reactor rats. A direct effect on the capillary wall may be important, as suggested by Gözsy and Kato (1957).

Further work is needed to study the action of antagonists of dextran and ovomucoid in reactor rats when the skin histamine is depleted, and it appears that through this type of experiment, progress will be made in deciding why non-reactor rats fail to react to dextran or ovomucoid. Non-reactivity to dextran in rats has recently been shown to be a genetically controlled character (Harris, Kalmus and West, 1963) so the problem may be of much wider application in the future.

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A SPECIFIC METHOD OF ASSAY OF STRONG SOLUTIONS OF BENZALKONIUM CHLORIDE AND OTHER QUATERNARY AMMONIUM GERMICIDES

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A method of assay of quaternary ammonium germicides in strong solutions or concentrated solid form is described. It can be used for solutions down to about 1 per cent in content. A known excess of iodide is added to the sample solution and the quaternary ammonium iodide is removed by shaking with chloroform. The excess iodide is titrated by an iodate method. When the chloroform extraction is made from a slightly alkaline solution only quaternary ammonium compounds are measured; if it is made from a slightly acid solution, non-quaternary cationic amine impurities are also included. The difference between assay results obtained from acid and alkaline extractions represents the non-quaternary amine content.

Several methods of assay exist for benzalkonium chloride and other similar quaternary ammonium germicides. Some of those designed for very dilute solutions are both convenient and selective, for example, the colorimetric method of Auerbach (1943), and adaptations of the titrimetric method of Barr, Oliver and Stubbings (1948), where solvent extraction is from an alkaline solution.

However, few satisfactory methods have been described for strong solutions such as the 50 per cent Benzalkonium Chloride Solution of the B.P.C. 1959, or the solid form of the U.S.P. XVI.

The available methods involve precipitation of an insoluble salt or complex, followed by filtration and determination of the excess precipitant, or gravimetric estimation of the precipitate. The precipitants include ferrocyanide (Lettermoser and Steudel, 1938), dichromate (Flotow, 1942), ferricyanide (Wilson, 1946), reineckate (Wilson, 1952, 1954), phosphotungstate (Yoshimura and Morita, 1955; Lincoln and Chinnick, 1956), phosphomolybdate (Yoshimura and Morita, 1955) and tetraphenylboron (Kirsten, Berggren and Nilsson, 1958). The Danish Pharmacopoeia (1954) adopts precipitation as the iodide in acid solution, then extracts the benzalkonium iodide with chloroform and titrates the separated chloroform solution with acetous perchloric acid. None of these methods distinguish the quaternary ammonium halide from tertiary long-chain alkyl amine hydro-halides which are the most likely organic impurities of the quaternary compound.

The ferricyanide method has been the one mostly used, having been the U.S.P. method since 1945. The B.P.C. has adopted a modified version. This method has proved its usefulness, but it is known to suffer from disadvantages. It involves filtration of the gelatinous benzalkonium ferricyanide which needs to stand for 1 hr., and a volume of the filtrate is titrated equivalent to half the volume of the precipitated mixture, thus

requiring a precipitate-volume correction. The B.P.C. version directs the complete washing of the precipitate free from ferricyanide, followed by titration of the whole of the filtrate. In addition, the end-point of the ferricyanide titration is not sharp and final, and the burette readings are small with the U.S.P. method while the B.P.C. employs a 5 g. sample, necessitating a volume of nearly 1 litre to be titrated to obtain a titration in the region of 20 ml.

The method to be described has been found satisfactory and convenient in routine use. It depends upon the ready extractability of benzalkonium iodide into chloroform in the presence of a small quantity of alkali. A known excess of potassium iodide solution is added to a dilution of the sample in a separator and the quaternary ammonium iodide removed by shaking with chloroform. In the alkaline conditions, any tertiary long-chain alkyldimethylamine hydrochlorides are converted to the amine bases having no effect upon the residual iodide which after acidifying with hydrochloric acid is then titrated in the separator with 0·05M potassium iodate solution by the method of Andrews (1903), with its sharp end-point. On a 1 g. sample of the 50 per cent solution, the final titration is about 15 ml., with a blank titration of approximately 30 ml.

In the presence of a small quantity of acid, tertiary cationic amine hydrochlorides are extracted as their hydriodides into the chloroform, giving high results. The difference between the titration readings of assays on the same quantity after both alkali and acid extractions quantitatively measures these non-quaternary amines (Table I).

TABLE I

COMPARISON OF TITRATION VOLUMES OBTAINED (BLANK MINUS TITRE) ON THE SAME APPROX. 2 PER CENT BENZALKONIUM CHLORIDE SOLUTION U.S.P. BY KIO_3 AND U.S.P. XVI METHODS, BEFORE AND AFTER ADDITION OF TERTIARY ALKYLDIMETHYLAMINE HYDROCHLORIDES. AVERAGE MOL. WT. 264, TO ASSAY MIXTURE

Method	KIO_3	KIO_3	U.S.P. XVI
Conditions	Alkaline (0·5 ml. N NaOH)	Acid (0·5 ml. N HCl)	—
Amount of sample soln. taken ..	25 ml.	25 ml.	50 ml.
Titrant	0·05 M KIO_3	0·05 M KIO_3	0·1 N $Na_2S_2O_3$
1. Sample soln. without addition	12·89 ml.	13·34 ml. (i)	4·38 ml.
2. As 1. with 48·5 mg. tertiary amine HCl's added	12·88 ml.	15·18 ml. (ii)	4·70 ml.
3. As 2. with 97·0 mg. tertiary amine HCl's added	12·89 ml.	17·04 ml. (iii)	5·04 ml. (iv)

(i) Non-quaternary amine HCl originally present calculated = 11·9 mg. (in 25 ml. of 2 per cent sample solution)
(ii) Added tertiary amine HCl recovered = 48·6 mg.
(iii) Added tertiary amine HCl recovered = 97·6 mg.
(iv) Added tertiary amine HCl recovered = 105 mg.

By applying the technique to pharmaceutical benzalkonium chloride solutions it should, therefore, be possible to detect the presence of and determine any such non-quaternary cationic amines. Examination of samples of varying origins showed these amines to form from almost nil

to about 2·5 per cent of the total weight as anhydrous benzalkonium chloride (Table II).

TABLE II

NON-QUATERNARY AMINE HYDROCHLORIDE CONTENTS FOUND IN PHARMACEUTICAL QUALITY BENZALKONIUM CHLORIDE SAMPLES OF VARIOUS ORIGINS, CALCULATED ON THE ANHYDROUS BENZALKONIUM CHLORIDE CONTENT

Sample	Acid and alkaline extraction titration difference, (A-B) ml. of 0·05 M KIO_3 on 25 ml. of a 2 per cent sample solution (ml.)	Non-quaternary amine HCl content (calc. on anhydrous) (per cent)
1	0·40	2·12
2	0·37	1·96
3	0·25	1·32
4	0·05	0·26
5	0·10	0·53
6	0·45	2·32

The method has been found to be accurate for crystalline, pure, benzyldimethylmyristylammonium chloride dihydrate, recrystallised from water until shown to be free from other long-chain alkyl homologues by paper chromatography, the moisture content being determined by drying *in vacuo* over phosphorous pentoxide giving an anhydrous mol. wt. of 368·0. Using the proposed method the result (mean of 14 determinations) was 91·03 $\pm$ 0·088 per cent while the U.S.P. method gave 90·87 $\pm$ 0·30 per cent. The assay figures obtained from both alkaline and acid iodide extractions were identical showing freedom from non-quaternary amines. The moisture content found was 8·92 per cent.

Samples of pure, tertiary long-chain alkyldimethylamines and their salts could be assayed by the method incorporating chloroform extraction of the hydriodides. From sufficiently alkaline extraction conditions, there was no iodide uptake. From slightly acid conditions, full recovery of the amines was obtained; more acid conditions gave slightly lower results. Table III gives results of a tertiary alkyldimethylamine hydrochloride analysis. A sample of pure primary dodecylamine hydrochloride

TABLE III

ANALYSIS OF AN APPROXIMATELY 96 PER CENT PURE, MIXED N-LONG CHAIN ALKYLDIMETHYLAMINE HYDROCHLORIDES, AVERAGE MOL. WT. 264, BY KIO_3 METHOD UNDER VARIOUS ALKALINE AND ACID EXTRACTION CONDITIONS

Wt. of amine HCl (100 per cent) taken for assay (mg.)	Acid or Alkali amount added (N)	Amine HCl found (mg.)
1. 100	0·5 ml. NaOH	Nil
2. 200	0·5 ml. NaOH	10·6
3. 200	1·0 ml. NaOH	Nil
4. 400	2·0 ml. NaOH	Nil
5. 400	0·5 ml. HCl	396

was also found to respond similarly to the two assay processes, except that only about 80–90 per cent recovery was obtained in the acid extraction of the hydriodide. This was also found to be influenced by the amount of excess acid and best recovery occurred near neutrality.

METHOD

Reagents

Potassium iodate 0·05M. Prepare a 0·05M solution by dissolving exactly 10·702 g. of potassium iodate (AR) previously dried at 105° for 1 hr. in water to 1000 ml.

Potassium iodide 5 *per cent.* Prepare w/v. Use only if colourless.

Method for Solutions and Solids Containing 30 *to* 100 *per cent Benzalkonium Chloride*

Accurately weigh a sample containing about 0·5 g. of anhydrous benzalkonium chloride, and transfer with the aid of water (35 ml.) to a 500 ml. glass-stoppered, conical separator containing chloroform (25 ml.). Add N sodium hydroxide solution (0·5 ml.) followed by the 5 per cent potassium iodide solution (exactly 10 ml.). Stopper the separator and shake well. Allow the two layers to separate and run off the lower chloroform layer through a loosely packed plug of about 0·5 g. of absorbent cotton wool,[1] placed in a small glass funnel, to absorb any traces of entrained aqueous liquid. (The chloroform filtrate, which can be discarded, should be clear and dry). Repeat the extraction with chloroform (3 × 10 ml.) running the chloroform layers each time through the same cotton wool filter. Finally, wash the filter with a further 5 ml. of chloroform, and allow the filter to drain.

Add to the separator, concentrated hydrochloric acid (40 ml.), preferably previously chilled,[2] and titrate the mixture in the separator with 0·05M potassium iodate until the solution becomes only light brown in colour; add chloroform (5 ml.), stopper, and shake. Continue the titration, with shaking, until the chloroform layer becomes colourless and the supernatant liquid is clear yellow. Remove the cotton wool filter from the glass funnel and add the cotton wool directly into the contents of the separator. Wash the glass funnel with water (2 or 3 ml.), receiving the washings in the separator. Stopper, shake, and complete the titration if necessary.

Perform a blank by titrating a mixture of water (20 ml.), 5 per cent potassium iodide solution (exactly 10 ml.), concentrated hydrochloric acid (40 ml.), and adding chloroform (5 ml.) when the mixture becomes light brown in colour.

The difference between the two titrations represents the 0·05M potassium iodate equivalent to the anhydrous benzalkonium chloride contained in the amount of solution taken for the assay.

Each ml. of 0·05M KIO_3 is equivalent to 0·0354 g. of anhydrous benzalkonium chloride, average mol. wt. 354[3].

[1] The use of the cotton wool filter to retain entrained aqueous liquid does not normally increase the burette reading by more than about 0·05 ml. No benzalkonium iodide remains adsorbed on to the cotton wool under the conditions described.

[2] The use of hydrochloric acid, chilled by storage of the stock bottle in a refrigerator reduces the temperature rise due to dilution of the acid. Otherwise, cool the mixture sufficiently, if necessary, before titrating.

Method for Solutions Containing 1 *to* 30 *per cent Benzalkonium Chloride*

Into a 500 ml. glass stoppered, conical separator containing chloroform (25 ml.), transfer an accurately measured volume, or weight, of the solution not exceeding 35 ml., containing as near as possible 0·5 g. of anhydrous benzalkonium chloride. If the volume of solution taken is less than 35 ml., add water to make the total aqueous liquid 35 ml. Proceed as above method commencing at the words: "Add N sodium hydroxide solution (0·5 ml.)."

Combined Assay Method and Determination of Non-quaternary Long-chain Alkylamine Hydrochlorides

Sample dilution: Transfer an accurately weighed amount, or accurately measured volume, of the sample, containing about 2 g. of anhydrous benzalkonium chloride, to a 100 ml. volumetric flask and dilute with water to 100 ml. Mix well.

Place in each of two glass-stoppered, conical separators labelled A and B respectively, 25 ml. of chloroform (25 ml.) and water (10 ml.). Transfer exactly 25 ml. of the sample dilution into each separator. To separator A add N sodium hydroxide solution (0·5 ml.); to separator B add N hydrochloric acid (0·5 ml.). To each separator add 5 per cent potassium iodide solution (exactly 10 ml.); stopper, and shake well.

Complete the assay method on each separator by the above method, commencing with the words: "Allow the two layers to separate . . ."

Let the burette reading (ml. 0·05M) of separator A be: A.

Let the burette reading (ml. 0·05M) of separator B be: B.

Let the burette reading (ml. 0·05M) of blank titration be: Blank.

Calculate the benzalkonium chloride content from 4(Blank—A) ml. which represents the anhydrous benzalkonium chloride contained in the original weight or volume taken to make the sample dilution.

Calculate the non-quaternary long-chain alkylamine hydrochlorides from 4(A — B) ml. which represents the non-quaternary amine hydrochlorides contained in the original weight or volume taken to make the sample dilution. Calculate the percentage found on the anhydrous benzalkonium chloride content.

Each ml. of 0·05M KIO_3 is equivalent to 0·0264 g. of non-quaternary long-chain alkylamine hydrochlorides, average mol. wt. 264.[3]

Discussion

In the alkaline assay extraction it is necessary to ensure that there is at least sufficient alkali present to ensure complete liberation of the amine bases from the hydrochlorides, otherwise some amine hydriodide will be extracted into the chloroform layer, as illustrated by example 2 of Table III. Reference to Tables II and III shows that 0·5 ml. N sodium hydroxide is sufficient for the quantity of non-quaternary long-chain alkyl amine hydrochlorides likely to be encountered in samples of pharmaceutical

[3] If the actual average molecular weight of the quaternary ammonium sample is known, then the equivalent weight used should be adjusted accordingly.

quality. Similarly, the amount of acid used must be sufficient to convert any free amine bases to their hydrochlorides; 0·5 ml. N hydrochloric acid is sufficient for about 100 mg. of bases. In general, the alkaline aqueous liquid extracted should be about pH 12. More strongly alkaline conditions can be used without affecting the result, but it is advisable to keep the amount of alkali used to a minimum to avoid neutralising some of the hydrochloric acid used in the final titration, as this generates heat. For the acid conditions, it is advisable to keep excess hydrochloric acid to a minimum, and on many samples less than 0·5 ml. of N hydrochloric acid can be used.

It may be noted that the specificity of the assay performed from alkaline extraction conditions parallels the finding of Auerbach (1943). In describing his colorimetric method for dilute quaternary solutions, in which the bromophenol blue dye salt of the quaternary is extracted by solvent from sodium carbonate solution, he pointed out that the basic medium gave the method its selectivity. Of about fifty non-quaternary amines which he tested, including dodecyldimethylamine, all responded negatively to his assay method.

The general iodide-iodate method was found to be applicable to cetrimide and cetylpyridinium halides.

It was also established experimentally that assay methods based on the Danish Pharmacopoeia (1954) method of non-aqueous perchloric acid titration of the chloroform extract, did not distinguish between quaternary and non-quaternary long-chain alkylamines when these are extracted from either acid or alkaline iodide solution. This is because both the non-quaternary amine hydriodides and bases respectively are extracted into the chloroform, and both titrate similarly with perchloric acid in non-aqueous conditions in the presence of mercuric acetate. Results are shown in Table IV.

TABLE IV

ACETOUS-PERCHLORIC ACID 0·1 N TITRATION OF CHLOROFORM EXTRACT FROM ACID AND ALKALINE CONDITIONS OF EQUIVALENT OF 0·675 G. ANHYDROUS BENZALKONIUM CHLORIDE, WITH AND WITHOUT ADDITION OF TERTIARY ALKYLDIMETHYLAMINE HYDROCHLORIDES, MEAN MOL. WT. 264

	Ml. 0·1 N Acetous-$HClO_4$ required	Added Amine HCls recovered
Extraction from acid conditions		
Sample	19·10	—
Sample + 27·2 mg. amine HCl ..	20·20	26·4 mg.
Extraction from alkaline conditions		
Sample	19·10	—
Sample + 27·2 mg. amine HCl ..	20·21	28·0 mg.

It was found, however, that the method could be made indicative of non-quaternary amine content of quaternaries by titrating the chloroform extract from the alkaline medium with acetous-perchloric acid to the first end-point before addition of mercuric acetate. When this end-point, indicating non-quaternary amine bases, is reached, mercuric acetate

solution is then added and the titration completed. The final end-point represents total amine and quaternary compounds. The first end-point was considered to be poor using Oracet Blue B or B.Z.L. Blue as indicator, presumably due to the small amount of amine bases involved and the presence of a large excess of quaternary iodide. The accuracy of attempts to determine the true quaternary content by difference by this method was thus affected.

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BLOCKADE OF 5-HYDROXYTRYPTAMINE ANTIDIURESIS IN RATS BY 2-BROMLYSERGIC ACID DIETHYLAMIDE TARTRATE AND 1-METHYL-LYSERGIC ACID BUTANOLAMIDE

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The effects of two lysergic acid derivatives, 2-bromlysergic acid diethylamide 'BOL 148' and 1-methyl-lysergic acid butanolamide 'UML 491' on the antidiuresis induced by 5-hydroxytryptamine '5-HT' has been studied in rats given a dose of 1 mg./kg. 5-HT subcutaneously. BOL 148, 5 mg./kg., and UML 491, 2·5 mg./kg., counteracted the induced antidiuresis. By contrast, the antidiuretic effect of posterior pituitary extract was not blocked or diminished by BOL 148 or by UML 491. BOL 148 and UML 491 alone did not have a diuretic or antidiuretic action.

5-HYDROXYTRYPTAMINE antagonists have been widely studied for their activity against various effects of the amine. The investigations on isolated smooth muscle preparations and the cardiovascular and respiratory systems have received the most attention (see, for example, Gaddum and Hameed, 1954; Outschoorn and Jacob, 1960; Venulet, 1961; Bunag and Walaszek, 1962).

The antagonism of 5-HT antidiuresis has attracted less interest. Dasgupta (1957) demonstrated that chlorpromazine antagonised the 5-HT-induced reduction of excretion. As chlorpromazine is an antagonist of 5-HT it was interesting to see if derivatives of lysergic acid acted similarly. The effects of BOL 148 and UML 491 on 5-HT antidiuresis are now reported.

MATERIALS AND METHOD

Seventy-two male white rats, 150 and 250 g., of a laboratory strain, fed with a standard diet and water *ad libitum*, were used. Food and water were withdrawn 12 hr. before the experiments. The rats were given a water load of 5 ml. of distilled water per 100 g. body weight through a stomach tube. They were kept 3 in a cage, the urine draining directly into a measuring cylinder below, and the volume recorded every 15 min. for 4 hr.

Rats which excreted less than 50 per cent of the water load in the first 100 min. were excluded from the experiments.

The drugs were given immediately after the water load: 5-HT as the creatinine sulphate 1 mg./kg. subcutaneously, BOL 148, 5 mg./kg. and UML 491, 2·5 mg./kg., intraperitoneally, posterior pituitary extract* 1 unit/kg. subcutaneously. Control groups received 0·9 per cent sodium chloride solution, 1 ml./kg., either s.c. or i.p. This had no influence on the urine excretion after the water load.

The room temperature varied between 16 and 18°. All experiments began at 9 a.m.

* Polfa.

RESULTS

The Influence of BOL 148 *and* UML 491 *on* 5-HT *Antidiuresis*

The arrangement of the experiments and the results are presented in Table I. The urine volume is expressed as a percentage of the water load.

TABLE I

URINE EXCRETION AFTER 5-HT ALONE AND WITH BOL 148 OR UML 491 GIVEN TO ANIMALS LOADED WITH WATER 5 ML./100 G.

Drug	Dose mg./kg.	Number of rats	Urine volume as percentage of the water load volume after			
			60 min.	120 min.	180 min.	240 min.
Control	–	18	41·0	71·8	80·4	83·2
5-HT	1	18	4·5	18·0	42·1	57·5
5-HT + BOL 148 ..	1 5	18	25·5	67·7	84·2	89·3
5-HT + UML 491 ..	1 2·5	18	31·4	62·3	76·4	80·2

The differences between the 5-HT group and the groups receiving 5-HT + BOL 148 and UML 491 are significant at the 5 per cent level.

Specificity of Antagonism

To prove the specificity of the antagonism of the 5-HT antidiuresis exerted by BOL 148 and UML 491, the experiments were repeated on the same rats using posterior pituitary extract given subcutaneously instead of 5-HT. The 5-HT antagonists have also been studied alone to ascertain whether they themselves have diuretic or antidiuretic properties. The relevant results are in Table II.

TABLE II

URINE EXCRETION AFTER BOL 148 OR UML 491 ALONE OR WITH POSTERIOR PITUITARY EXTRACT GIVEN TO ANIMALS LOADED WITH WATER 5 ML./100 G.

Drug	Dose mg./kg.	Number of rats	Urine volume as percentage of the water load volume after			
			60 min.	120 min.	180 min.	240 min.
BOL 148	5·0	12	32·4	73·5	84·0	88·8
UML 491	2·5	12	39·5	66·3	86·1	88·4
Post. pituitary extract	1 unit/kg.	12	14·1	22·1	36·1	41·7
Post. pituitary extract + BOL 148	1 unit/kg. 5·0	12	11·0	23·2	40·3	42·0
Post. pituitary extract + UML 491	1 unit/kg. 2·5	12	13·8	24·1	37·3	46·0

The volume of urine excreted at 240 min. showed a significant variation between the groups receiving posterior pituitary extract, posterior pituitary extract + BOL 148, posterior pituitary extract + UML 491 and the control group from Table I.

The results were also evaluated by calculating the point at which half of the total urine quantity was excreted, "the point of maximum excretion rate" (Burn, Finney and Goodwin, 1952). It was considered unnecessary to reject an "initial urine amount not belonging to the true period of

excretion", as recommended by Burn and others, if the urine bladder was previously emptied. The results are given in Table III.

TABLE III

THE POINT OF MAXIMUM RATE OF EXCRETION (MIN.) AFTER A WATER LOAD OF 5 ML./100 G.

Drugs	Dose mg./kg.	Point of maximum rate of excretion in different groups of 3 rats Average (min.)
Control	—	62·6 (54–71)
5-HT	1·0	149·7 (135–171)
5-HT + BOL 148	1·0	85·0 (71–95)
	5·0	
5-HT + UML 491	1·0	78·6 (70–94)
	2·5	
UML 491	2·5	66·2 (57–78)
BOL 148	5·0	74·2 (57–84)
Posterior pituitary extract	1 unit/kg.	112·7 (96–138)
Posterior pituitary extract + BOL 148	1 unit/kg.	106·7 (90–128)
	5·0	
Posterior pituitary extract + UML 491	1 unit/kg.	117·5 (91–135)
	2·5	

DISCUSSION

The data in Tables I and III show that in the hydrated rats a single injection of 5-HT, 1 mg./kg., causes a reduction of the urine excretion. A simultaneous intraperitoneal injection of BOL 148 or UML 491 inhibits or diminishes this antidiuretic action. This is evident both when comparing the hourly recorded urine volume in the groups and when estimating the points of maximum rate of excretion.

In considering how BOL 148 and UML 491 act against a 5-HT antidiuresis it is necessary to recall the theories of the mode of action of 5-HT on the kidneys. Erspamer (1956) first concluded that the 5-HT antidiuresis was due to preferential constriction of the afferent glomerular arterioles. Sala and Castegnaro (1953), on the other hand, believe that 5-HT is a water reabsorbing hormone. The vascular origin of the antidiuretic action of 5-HT was confirmed by Abrahams and Pickford (1956). Thus it seems probable that the lysergic acid derivatives abolish or diminish the antidiuretic response to 5-HT in the same manner as they prevent its cardiovascular effects.

The specifity of this action was investigated, and it was found that antidiuresis after posterior pituitary extract was not prevented by BOL 148 and UML 491. Neither BOL 148 nor UML 491 have significant diuretic or antidiuretic properties. Therefore it is concluded that their action on 5-HT antidiuresis is a specific one.

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A NOTE ON THE FACTORS AFFECTING R_F VALUES ON CITRATE BUFFERED PAPER CHROMATOGRAMS

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Experiments have been made to determine the effect of changes in experimental conditions on the speed of running of citrate/butanol systems on paper chromatograms.

Paper chromatography is of the greatest value for the provisional identification of unknown nitrogenous bases in toxicological analysis. Among the systems widely used for this purpose are those using buffered paper such as the citrate-butanol method of Curry and Powell (1954). Such systems, however, suffer from the disadvantage that the reproducibility of the R_F value is poor, particularly from one laboratory to another (McKee, 1957) and attempts to improve it by use of freshly prepared solutions, by equilibration, and by control of other variable factors are of doubtful value (Goldbaum and Kazyak, 1956) and have the disadvantage that they entail sacrifice of the three outstanding advantages of paper chromatography: speed, simplicity and cheapness.

In the course of some thousands of runs made with the citrate-butanol system it was noticed that certain factors had a considerable influence on the speed of running while others made little difference. A series of experiments was made to confirm these observations. It is thought that the results obtained are worth recording.

Experimental

For these experiments, unless otherwise stated, the solvent consisted of the organic rich layer left after shaking butanol with its own volume of 2 per cent aqueous citric acid. Sheets of No. 1 Whatman filter paper (14 in. × 6 in.) were dipped in a 5 per cent solution of sodium dihydrogen citrate, blotted, dried for 1 hr. at 25° in a fume cupboard with the draught on and stored in a folder until required. The ascending method was used, the tank being 11 in. × $8\frac{1}{2}$ in. × $15\frac{1}{2}$ in. high. In the equilibration experiments a magnetic device was used to hold the sheets above the solvent until it was required to lower them. The alkaloids used were cocaine, (−)-hyoscyamine, morphine, strychnine and coniine. For the major factors confirmatory experiments were made using brucine, hyoscine, antazoline, diphenhydramine, nikethamide, amylocaine, and naphthazoline.

Results and Discussion

Equilibration is undesirable because in addition to being time consuming it makes most substances run faster. For example, without equilibration the R_F values were cocaine 0·42, hyoscyamine 0·37, morphine 0·14,

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strychnine 0·28, coniine 0·60 and after equilibration for 18 hr. 0·66, 0·60, 0·22, 0·52, 0·64 respectively. The R_F values of some 500 alkaloids (Clarke, 1962) were found to show the following distribution. R_F values are given first with the number of alkaloids in parentheses. 0 (13); 0–0·1 (43); 0·11–0·20 (47); 0·21–0·3 (49); 0·31–0·4 (47); 0·41–0·5 (61); 0·51–0·6 (68); 0·61–0·7 (96); 0·71–0·8 (49); 0·81–0·9 (22); 0·91–1·0 (13). Thus any procedure which increases the speed of running tends to crowd still further the upper half of the scale. This results in loss of resolution, and increases the difficulty of identification by R_F value.

Fresh solvent solutions gave faster and more erratic running than those which had been used for some time. With freshly prepared solvent the values were cocaine, 0·53; hyoscyamine, 0·50; morphine, 0·23; strychnine, 0·43; coniine, 0·63, but with an old solution these were 0·43, 0·38, 0·14, 0·29, 0·60. This was further investigated by using mixtures made by adding different volumes of water to a 0·48 per cent solution of citric acid in butanol, and it was found that there was a direct connection between the speed of running and the water content of the solvent. The results with the 5 alkaloids in the order given above were:

Water added, per cent	S.G.	R_F values
4	0·821	0·18, 0·18, 0·09, 0·10, 0·53
8	0·830	0·33, 0·31, 0·12, 0·23, 0·57
12	0·838	0·43, 0·35, 0·14, 0·26, 0·60
16	0·846	0·41, 0·38, 0·14, 0·26, 0·64
Saturated with water layer	—	0·54, 0·43, 0·17, 0·34, 0·64
Saturated with water layer equilibrated overnight..	—	0·69, 0·64, 0·25, 0·59, 0·64

There is little change between a water content of 12 and 16 per cent. The measurement of specific gravity forms a reliable guide to the composition of the solution and any drop may be corrected by adding water.

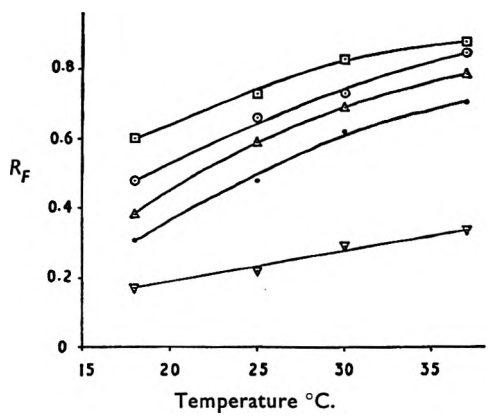

FIG. 1. R_F values plotted against temperature. ⊡ coniine, ⊙ cocaine, △ (−)-hyoscyamine, ● strychnine, ▽ morphine.

Increase of temperature, as is well known, increases the R_F values. The order of magnitude for this system is shown in Fig. 1.

The *time factor* was investigated by using fluorescent alkaloids and an ultra-violet lamp to determine the approximate R_F values at various stages during a run. There is a slow but steady increase of R_F value with time (Fig. 2). This is borne out by the higher values obtained after all-night runs.

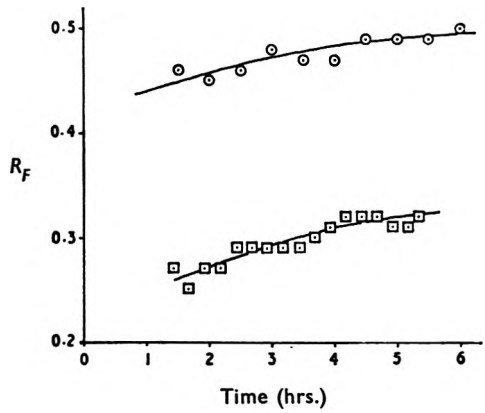

FIG. 2. R_F values plotted against time. ⊙ quinine, ⊡ proflavine.

The number of sheets in the tank had a profound effect on the R_F values. With a single sheet the values were cocaine, 0·57; hyoscyamine, 0·52; morphine, 0·21; strychnine 0·45; coniine, 0·65, but with four sheets the values were 0·42, 0·38, 0·15, 0·28, 0·62.

Tank size had a similar effect. For example, with a single sheet in a normal tank the R_F values for the same 5 alkaloids were 0·54, 0·48, 0 19, 0·41, 0·63, but with a single sheet in a small tank ($5\frac{1}{2}$ in. × $3\frac{1}{2}$ in. × $12\frac{1}{2}$ in. high) they were 0·39, 0·34, 0·14, 0·26, 0·58. If the system has been equilibrated the size of the tank and the number of sheets in it have no effect.

The above factors have the most influence on R_F values. No appreciable change in R_F value was caused by varying the concentration of the alkaloid solution, by the lateral position of the spot on the paper provided it was more than 1 cm. from the paper edge, nor by the distance of the starting line above the surface of the liquid. "Mixed spots" of alkaloids ran at the same speed as individual spots, while saturating the solvent with butyl citrate had no effect. Wet spots ran faster than dry ones and these again ran slightly faster than those dried with hot air. Paper kindly prepared for us by Dr. A. S. Curry gave similar results to paper prepared in this laboratory.

It may be concluded that reasonable reproducibility of R_F values may be obtained without loss of either resolution or simplicity if allowance is made for the factors outlined. It is worth noting how much less the R_F value of coniine varies with experimental conditions than do the R_F values of the other alkaloids used. This emphasises the danger of expressing the R_F value of one alkaloid in terms of the R_F value of another.

As the solvent should not be saturated with water it may be prepared conveniently by dissolving 4·8 g. of citric acid in a mixture of 160 ml. of water and 840 ml. of butanol, the specific gravity being kept between 0·838–0·846 by the addition of water.

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THE ANATOMY OF *DIOSCOREA BELIZENSIS* LUNDELL

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A steroid-yielding wild yam from British Honduras is shown to be *Dioscorea belizensis* Lundell. The macroscopical characters of the entire plant and the microscopical characters of the underground organs are described.

IN 1941 Lundell found and described *Dioscorea belizensis* Lundell. His description was not illustrated and made only passing references to the underground organs. Lundell stated that pistillate flowers and fruits were unknown; none have since been found. A description of this species seemed desirable owing to its possible use as a commercial source of diosgenin (Blunden, 1962; Blunden and Hardman, 1963).

Habitat

D. belizensis was found growing in a five year old *Pinus caribaea* Morelet plantation at the Machaca Forest Station in British Honduras by Dr. S. S. Bampton of the Tropical Products Institute, London. The plant was only moderate in occurrence beyond the Forest Station, which is located in southern British Honduras between the Rio Grande and River Moho and about 15 miles inland from Punta Gorda.

The temperature of the region remains fairly constant throughout the year, the mean maximum varying between 23° in January and 31° in April, and the mean minimum between 14° in January and 22° in June. Apart from the two coldest months of December and January the mean maximum for 1960 varied by only 3° and the mean minimum by 4°.

The rainfall for the Machaca Forest Station is high. Of the total of 313 cm. for 1960, 201 cm. fell in the four months of June, July, August and September, and only 38 cm. in the four months from December to March.

Materials

All the plant material received from British Honduras was collected from the Machaca Forest Station by Mr. R. Waters, the Conservator of Forests, and despatched by air mail. Batches of tuber were received in October, 1960, January, February, April, May, July, August, and October, 1961. Aerial stems and leaves were received in January, February and October, 1961, and a male inflorescence was received in January, 1961. Other plants were grown in the Department of Pharmacy's hot-house at Nottingham from pieces of tuber received from British Honduras.

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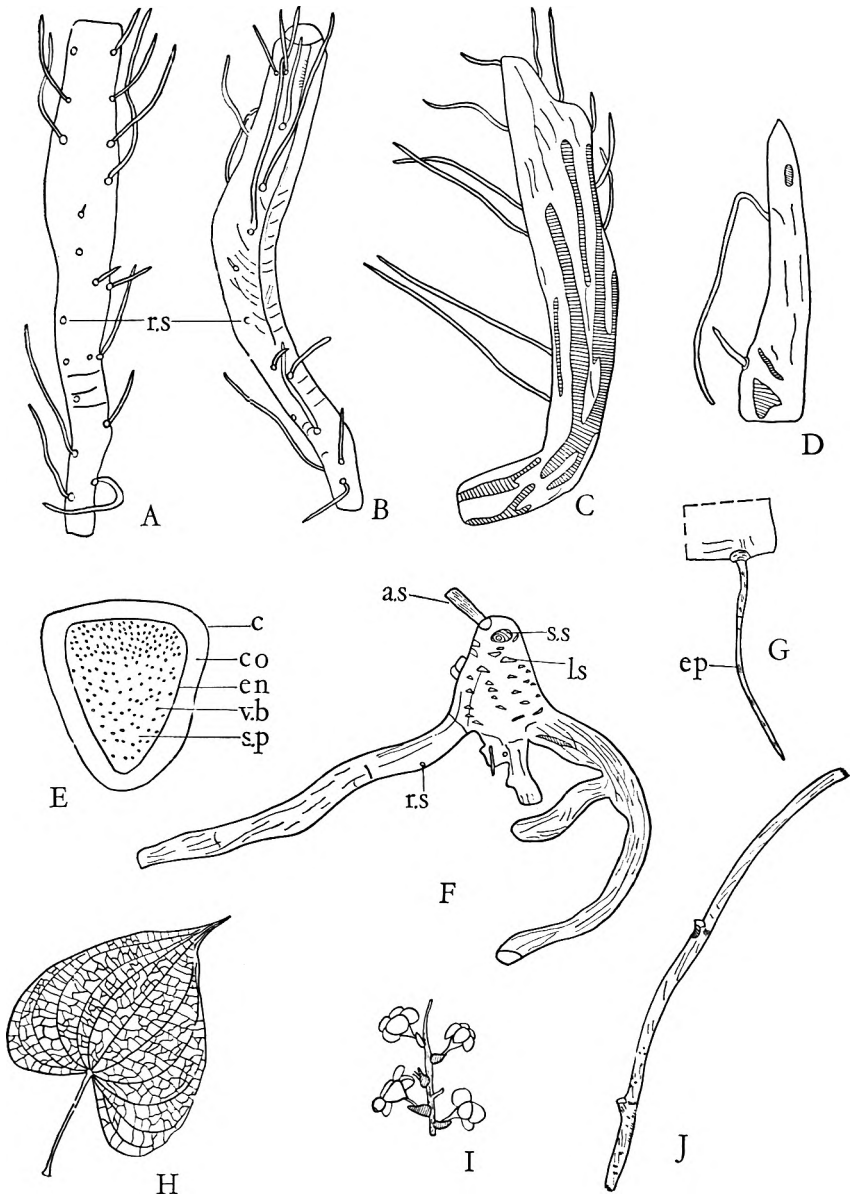

FIG. 1. *Dioscorea belizensis* Lundell. A piece of tuber: A, lower surface; B, side view; C, upper surface; D, end of a tuber branch; E, transverse section of a tuber branch; F, crown of tuber; E × ¾, the rest by ½. G, root × ½. H, leaf × ¼. I, part of male inflorescence × 2. J, piece of stem × ¼. *a.s.*, aerial stem; *c.*, cork; *co.*, cortex; *en.*, endodermis; *ep.*, epidermis; *l.s.*, leaf scar; *r.s.*, root scar; *s.p.*, stele parenchyma; *s.s.*, stem scar; *v.b.*, vascular bundle.

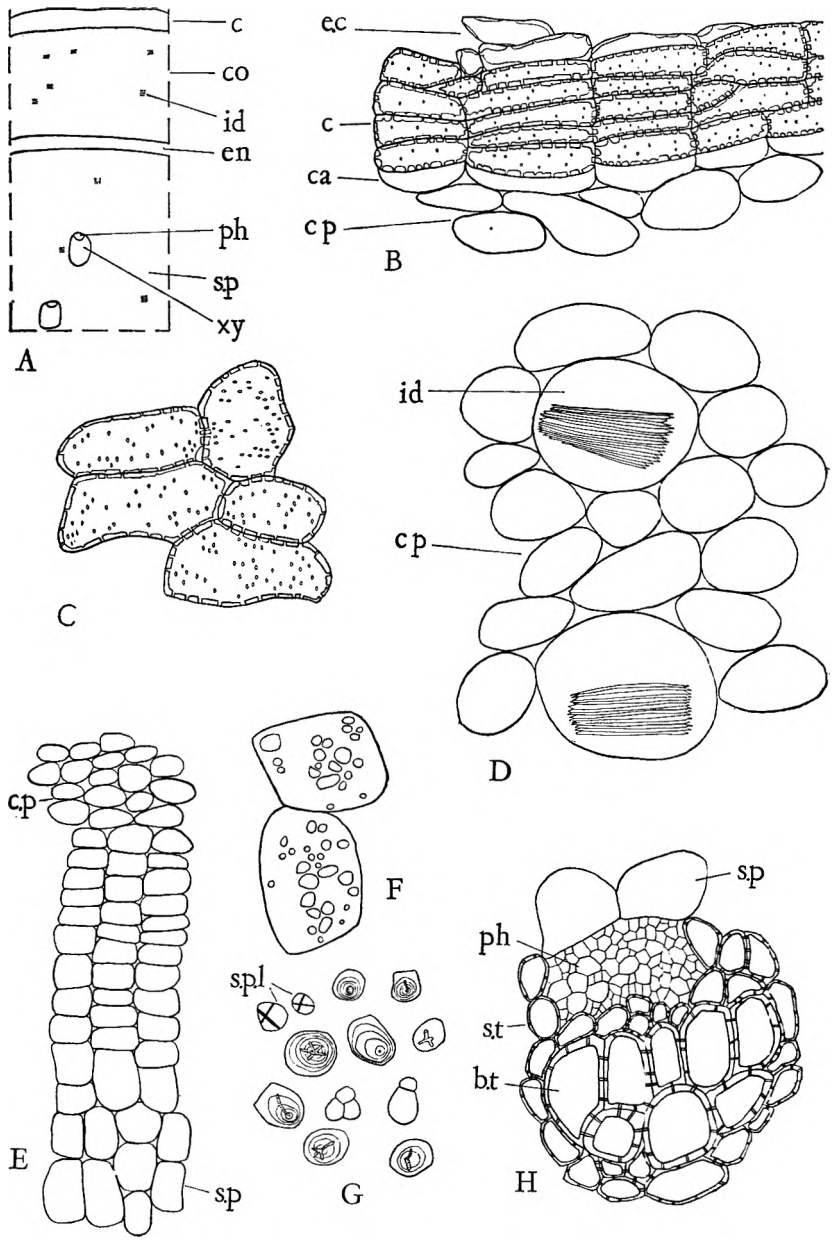

FIG. 2. Tuber of *Dioscorea belizensis* Lundell. A, transverse section, general diagram. B, transverse section of outer tissues. C, surface view of cork. D, transverse section of cortex. E, transverse section through the endodermal region. F, parenchyma cells containing starch grains. G, starch grains. H, transverse section through vascular bundle. A × 20, E × 60, the rest × 210. *b.t.*, bordered pitted tracheids; *c.*, cork; *ca.*, phellogen; *co.*, cortex; *c.p.*, cortical parenchyma; *e.c.*, exfoliating cells; *en.*, endodermal region; *id.*, idioblast; *p.*, parenchyma; *ph.*, phloem; *s.p.*, stele parenchyma; *s.p.l.*, starch under polarised light; *s.t.*, simple pitted tracheids; *xy.*, xylem.

The Plant

D. belizensis is a perennial vine with a large tuber, conical at the top and bearing a few cylindrical branches which grow horizontally about 30 cm. below the surface of the ground. In February and March, the two driest months of the year, *D. belizensis* has a resting stage, when growth is restricted to new leaves. In exceptional cases, however, as when the vine is damaged, a new shoot is stimulated into growth. The plant flowers infrequently, usually in October and November after the rainy season and preceding the two cooler months.

Macroscopical Characters

The plant has an AERIAL STEM, twining dextrorsely to heights of up to 3·7 m., which may be between 9 m. and 13·5 m. in length and up to 11·5 mm. in diameter, although it is usually less than 6 mm. Stems from British Honduras plants were woody, fibrous, tough and broke with a fibrous fracture. Younger plants grown at Nottingham under greenhouse conditions had herbaceous stems. The stems have rough surfaces, are ridged longitudinally and transversely, the transverse ridges being confined to the lower part. The thin cork separates easily from the older regions (Fig. 1, J).

THE LEAVES are alternate and often absent from the lower half of the vine. They are petiolate, the petiole being 5 to 26·5 cm. long and up to 3 mm. in diameter. The colour varies from a brownish-green in the young to a dark brown in the older leaves. The petiole has a pulvinus at each end and bears longitudinal ridges which are continuous with the main veins of the lamina. The surface is sparsely lepidote (Fig. 1, H).

The lamina is simple, broadly ovate, up to 27 cm. long and 25·5 cm. wide, margin entire, apex abruptly cuspidate, base deeply cordate with a broad open sinus, eleven veined, of which only three reach the apex of the leaf and with the outer veins branching from the penultimate ones, the veins and veinlets being impressed above and very prominent below and with reticulate venation. The upper surface is glabrous and the lower shortly villous. The lamina varies in colour from green to a dark brownish-green and has a papery texture, particularly in the older leaves.

THE FLOWER. The staminate inflorescences are axillary, solitary, up to 150 cm. long and consist of a narrow panicle of racemes up to 7·5 cm. in length. No pistillate flowers or fruits were received. The rachis and peduncle are both longitudinally ridged and somewhat woody. The surface is sparsely lepidote. The bractlets subtending the flowers are up to 2 mm. long, ovate, abruptly cuspidate and, like the flowers, are dark red in colour. The flowers are actinomorphic. The pedicels are from 1 to 2 mm. long and sparsely lepidote. The perianth has six lobes, each lobe being broadly ovate and about 2 mm. long. There are six stamens which are attached to the bases of the perianth lobes. The filaments are thick and about 0·5 mm. long. The anthers are about 0·2 mm. in length, bilocular and introrse and are not appreciably wider than the filaments. The inferior ovary is abortive, but shows a trilocular structure (Fig. 1, I).

THE TUBER constitutes the greatest part by weight of the plant, the average being 3 kg. from each plant from British Honduras. The tuber has a conical crown, with a vertical axis (Fig. 1, F). The cone varies in size from 1·5 to 7 cm. high and 1·2 to 5·5 cm. in diameter at the base. From the top of the cone grows the aerial stem (Fig. 1, F). From the base of the crown emerge from three to eight branches which run horizontally under the ground and often reach considerable lengths. Most of the tubers from British Honduras were in broken lengths, the longest was 75 cm. and the diameters vary from 0·6 to 5 cm. The horizontal branches are often bifurcated, usually near to their junction with the crown.

Near the apex of the crown there are usually one or two large depressed scars, up to 1·5 cm. in diameter, produced by previous stems. The conical crown bears several large brown triangular scale leaves up to 1·3 cm. broad (Fig. 1, F).

The older parts of the tuber including the crown are an orange-brown, the orange colour being most pronounced in the younger parts. The tuber is somewhat triangular in transverse section, the upper surface being quite frequently markedly flattened, particularly in the older lengths. The adventitious roots arise in regular longitudinal rows on the sides of the tuber; circular root scars, up to 4·5 mm. in diameter, are to be found (Fig. 1, A). The upper surface and the upper half of the sides of the tuber frequently bear marked longitudinal furrows in the cork (Fig. 1, B and C), but the lower surface and the lower half of the sides usually have a smooth surface. When the tuber has partially dried out, which sometimes occurs when received from British Honduras, very prominent longitudinal furrows are apparent.

The fresh tuber is flexible but breaks fairly easily, the resistance to breaking being mainly due to the cork; it has no marked odour and possesses a bitter, unpleasant taste. The transversely cut surface shows a thin cork, which is easily peeled from the tuber, a narrow yellowish-cream cortex, a dark band marking the endodermal region, and a large yellowish stele, in which are scattered numerous vascular bundles (Fig. 1, E).

THE ROOT. The roots are adventitious; they are usually less than 12 cm. but may be up to 25 cm. in length, and usually 2 to 3 mm. in diameter near their junction with the tuber (Fig. 1, G). The root is a light brown colour, with paler brownish-fawn areas where the epidermis has exfoliated; the cortex is brittle and easily separates from the inner tissues exposing the endodermis and giving rise to dark brown areas. Along the length of the roots, particularly those from British Honduras, there are transverse cracks in the cortex. The root has a dry powdery texture, breaks easily with a porous fracture, does not possess any marked odour and is tasteless. The transversely cut surface shows a thin epidermis, a wide brownish-fawn lignified cortex, a dark brown lignified endodermis and a brownish-fawn stele, which except for the phloem, is lignified; the vascular bundles have the normal monocotyledonous arrangement (Fig. 4, A).

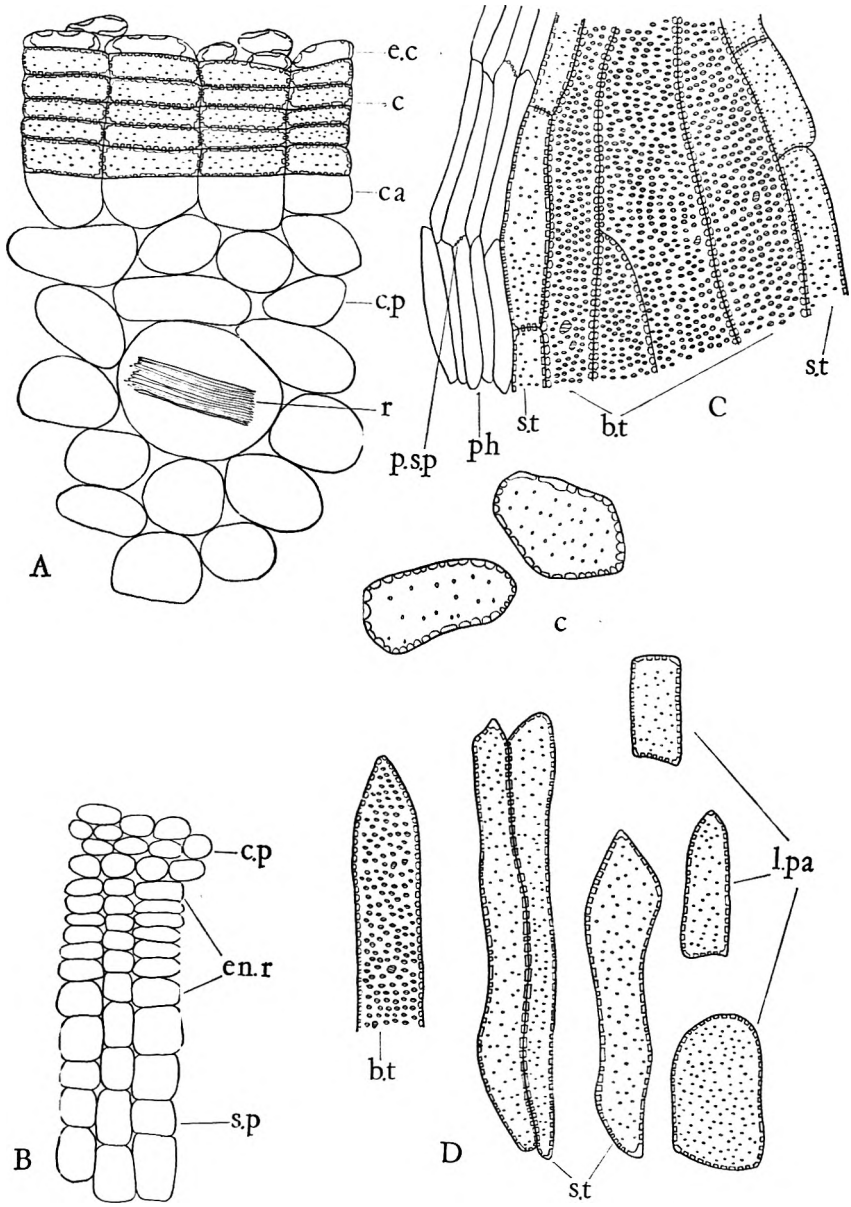

FIG. 3. Tuber of *Dioscorea belizensis* Lundell. Longitudinal sections through: A, the outer tissues; B, the endodermal region; C, vascular bundle. D, lignified cells isolated by maceration. B × 60, the rest × 210. *b.t.*, bordered pitted tracheid *c.*, cork; *ca.*, phellogen; *c.p.*, cortical parenchyma; *e.c.*, exfoliating cells; *en.r.*, endodermal region; *l.p.*, lignified parenchyma; *ph.*, phloem; *p.sp.*, sieve plate; *r.*, raphide of calcium oxalate; *s.p.*, stele parenchyma; *s.t.*, simple pitted tracheids.

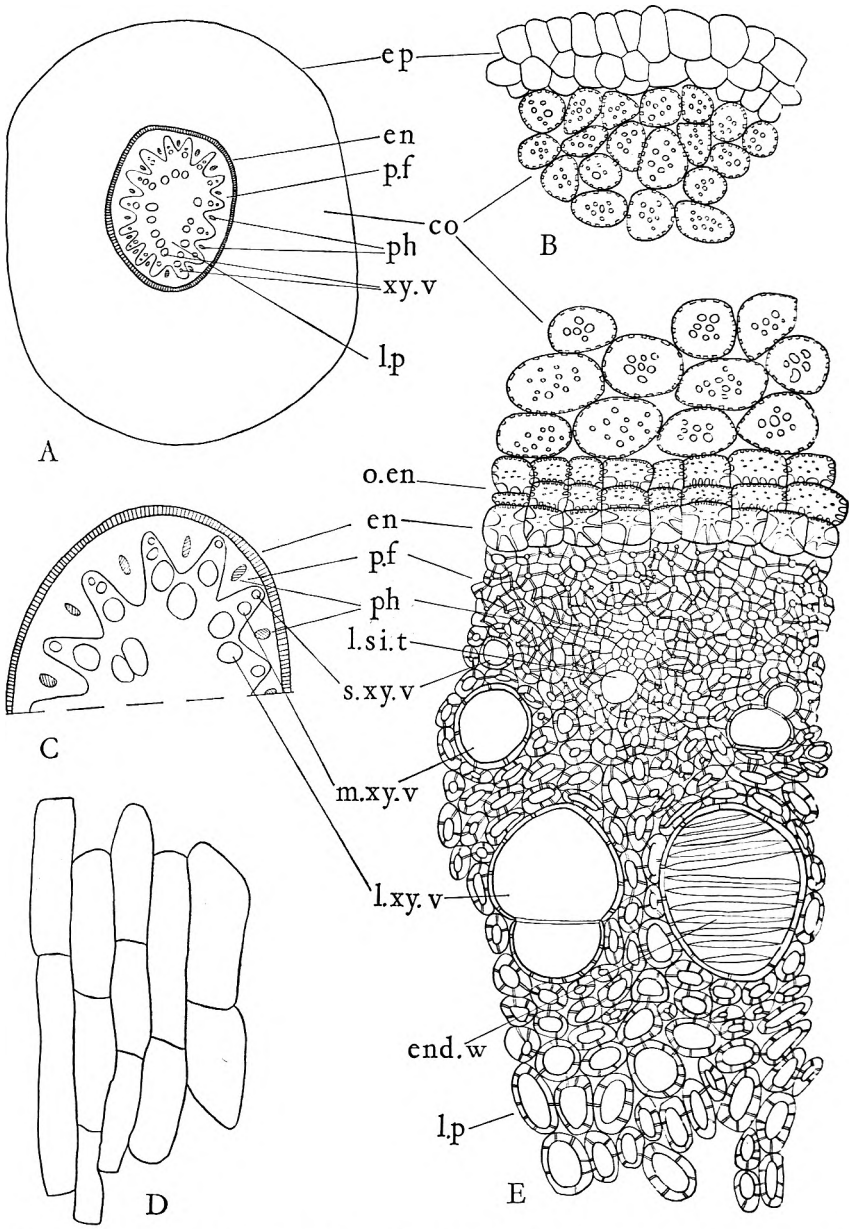

FIG. 4. Root of *Dioscorea belizensis* Lundell. A, transverse section, general diagram. B, transverse section through outer tissues. C, transverse section of stele, general diagram. D, surface view of epidermis. E, transverse section of inner tissues. A × 28, C × 52, the rest × 210. *co.*, cortex; *en.*, endodermis; *end.w.*, end wall of vessel; *ep.*, epidermis; *l.p.*, lignified parenchyma; *l.si.t.*, large sieve tube; *l.xy.v.*, large xylem vessel; *m.xy.v.*, medium xylem vessel; *o.en.*, outer endodermis; *p.f.*, pericyclic fibres; *ph.*, phloem; *s.xy.v.*, small xylem vessel (protoxylem); *xy.v.*, xylem vessel.

Microscopical Characters

THE TUBER. The outer protective layer of the tuber is composed of a number of very regularly arranged rows of lignified cork cells (Figs. 2, B and 3, A); usually between four and nine, but there may be as many as thirty-five. Of these rows only up to thirteen appear to be completely functional, the outer layers being in the process of breaking down and exfoliating; outside the cork, there is usually a layer of broken-down, brown coloured cells, which are often non-lignified (Figs. 2, B and 3, A).

The individual cork cells are straight-walled and usually five or six sided in surface view (Fig. 2, C), in transverse and longitudinal section they appear rectangular (Figs. 2, B and 3, A), individual cells being elongated tangentially, and some also elongated longitudinally. The walls are thick, lignified and cuticularised and have frequent simple pits. The cells measure T = 28–**60**–**114**–123 μ, L = 24–**36**–**63**–81 μ and R = 10–**12**–**23**–32 μ.

Under certain conditions a second phellogen arises deep in the cortex, producing lignified cells to the outside. The parenchymatous cortical cells thus cut off then gradually become pigmented and eventually are exfoliated along with the outer cork layer. Cork layers are produced also to cover cut or damaged surfaces of the tuber.

The cortex consists of cellulose-walled parenchymatous cells, oval in both longitudinal and transverse section (Figs. 2, D and 3, A), of varying dimensions: T = 35–**60**–**120**–162 μ, L = 42–**54**–**110**–145 μ and R = 15–**30**–**60**–80 μ.

Idioblasts containing raphides of calcium oxalate are found in the cortex and stele, but occur more frequently in the cortex. Although they have the same shape as the surrounding cells the idioblasts are much larger with dimensions: T = 87–**105**–**147**–168 μ, L = 84–**100**–**155**–180 μ and R = 65–**75**–**105**–138 μ. The calcium oxalate raphides measure: T = 60–**68**–**96**–135 μ and width 20–**30**–**45**–48 μ. These are surrounded by material which is stained red by corallin-soda and orange-brown with iodine solution suggesting mucilage.

No endodermis is distinguishable, but there is a region which appears darker than the surrounding layers and contains less starch than the cortical cells or the tissues of the stele. In this endodermal region the parenchyma consists of several layers of regularly arranged, straight-walled, rectangular cells which measure: T = 48–**63**–**114**–135 μ, L = 60–**100**–**140**–165 μ, and R = 18–**30**–**70**–108 μ. Towards the centre of the stele the cells show a gradual radial elongation, as opposed to the tangential elongation of the cortical cells (Figs. 2, E and 3, B).

The ground tissue of the stele consists of radially elongated parenchyma; the cell dimensions are: T = 45–**70**–**110**–144 μ, L = 75–**90**–**140**–156 μ. Radially most of the cells are in excess of 100 μ and are up to 230 μ towards the centre of the tuber.

Starch grains (Fig. 2, F and G) are found in the parenchyma of both the cortex, where they are few, and stele, where they are abundant. Most of the grains are simple and a few 2- to 3-compound; individual granules have blunt angles or are more or less rounded with an eccentric

hilum which appears as a circular spot or frequently as a simple curved or multiple cleft. Striations are often clearly visible and individual grains are up to 35 μ in diameter, although about 40 per cent are less than 10 μ.

No protein bodies or fat crystals were seen in sections of the tuber, but sections were very strongly stained with tincture of Alkanna, picric acid, and Millon's reagent particularly those from the cortical region.

Scattered throughout the stele are numerous collateral vascular bundles The phloem consists of longitudinally elongated, cellulose-walled sieve tubes, the sieve areas occurring in the end walls of the cells (Fig. 3, C). The xylem consists of a central core of long, wide-diameter tracheids, having thick lignified cell walls with numerous bordered pits having very marked extended pit apertures. The outer zone of the xylem consists of lignified parenchyma and tracheids, both of which have relatively thick, lignified walls with simple pits. This zone contains cells which show gradual changes from wide diameter lignified parenchyma to narrow diameter lignified tracheids (Figs. 2, H, 3, C and D). The cell dimensions of the outer zone of the xylem cover a wide range and there is a gradual transition between lignified parenchyma and the tracheids; the dimensions are: L = 60–**90**–**270**–480 μ, and width 9–**24**–**57**–78 μ. The central zone of tracheids with bordered pits have thicker walls than the simple pitted tracheids; they also have a wider diameter, i.e. 21–**48**–**87**–111 μ and a much greater length, being almost invariably in excess of 500 μ.

THE ROOT. The epidermis of the root together with one to four subjacent rows have yellowish-brown, suberised walls. The individual cells are roughly hexagonal to rectangular in transverse section (Fig. 4, B), but they are elongated longitudinally (Figs. 4, D and 5, A) measuring L = 39–**75**–**162**–220 μ, R = T = 8–**21**–**39**–51 μ. The epidermis is fragile and on many of the roots received from British Honduras was absent or present in a broken-down form.

The cortex is a wide band and accounts for about two-thirds of the root diameter. The individual cells are circular or oval in transverse section, but are more elongated longitudinally, this being most pronounced in the outer layers of the cortex (Fig. 5, A); L = 21–**33**–**75**–228 μ, T = 18–**36**–**60**–72 μ, and R = 18–**30**–**48**–63 μ. The cells have relatively thin, slightly lignified cell walls with very large simple pits (Figs. 4, B, 5, A and B).

The endodermis consists of a single layer of deep brown, longitudinally elongated cells. To the outside, are one or two layers of cells which have a somewhat similar form to those of the endodermis, but the cells are not usually so large and have less thickened walls (Figs. 4, E and 5, B). The endodermal cells (Fig. 4, E) measure: L = 70–**100**–**186**–270 μ, R and T = 14–**24**–**36**–45 μ; they have thick, simple pitted walls, the inner and radial walls being considerably more thickened than the outer walls (Figs. 5. B and 6, D).

Within the endodermis is a pericycle composed mainly of yellow, strongly lignified fibres, with a few yellow stone cells; this layer forms a band within the endodermis and extends between the different xylem

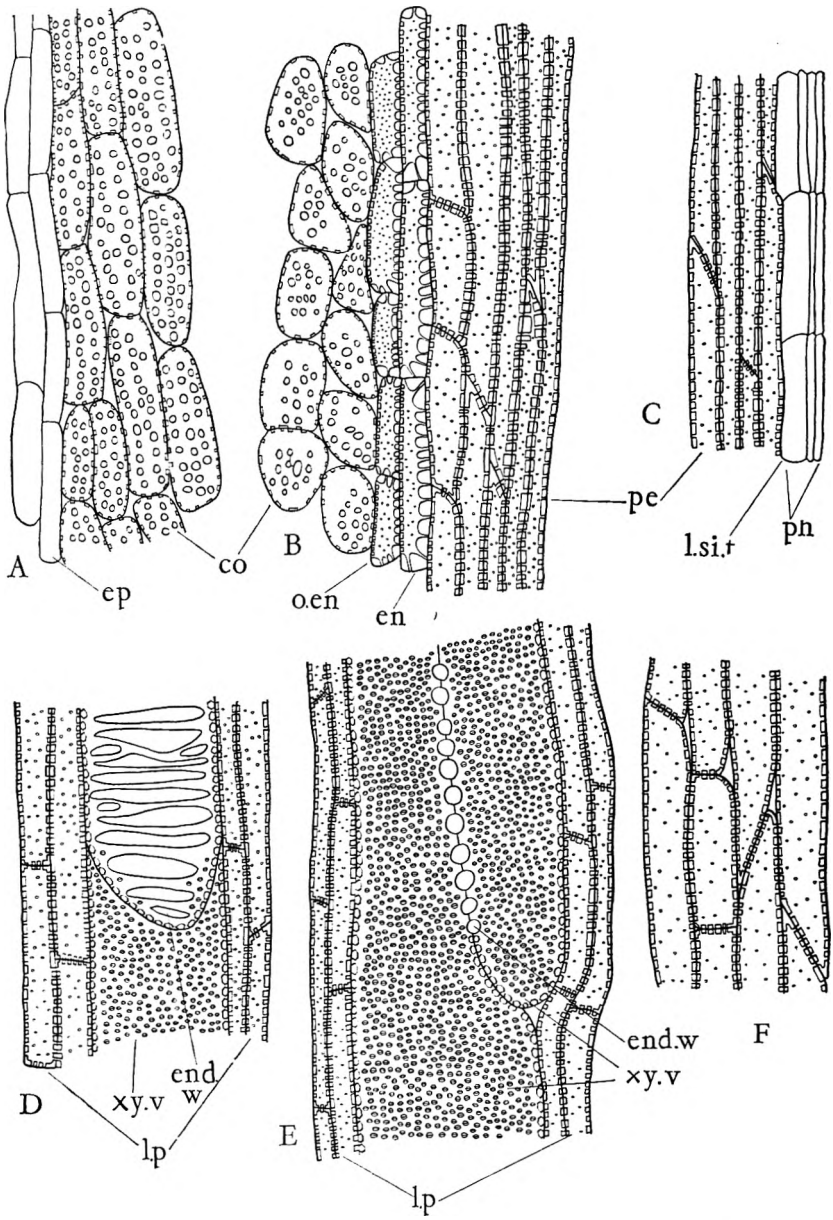

FIG. 5. Root of *Dioscorea belizensis* Lundell. Longitudinal sections: A, outer tissues. B, endodermal region. C, phloem tissue. D and E, xylem areas. F, lignified parenchyma. All × 210. *co.*, cortex; *en.*, endodermis; *end.w.*, end wall; *ep.*, epidermis; *l.p.*, lignified parenchyma; *l.si.t.*, large sieve tube; *o.en.*, outer endodermis; *pe.*, pericycle; *ph.*, phloem; *xy.v.*, xylem vessel.

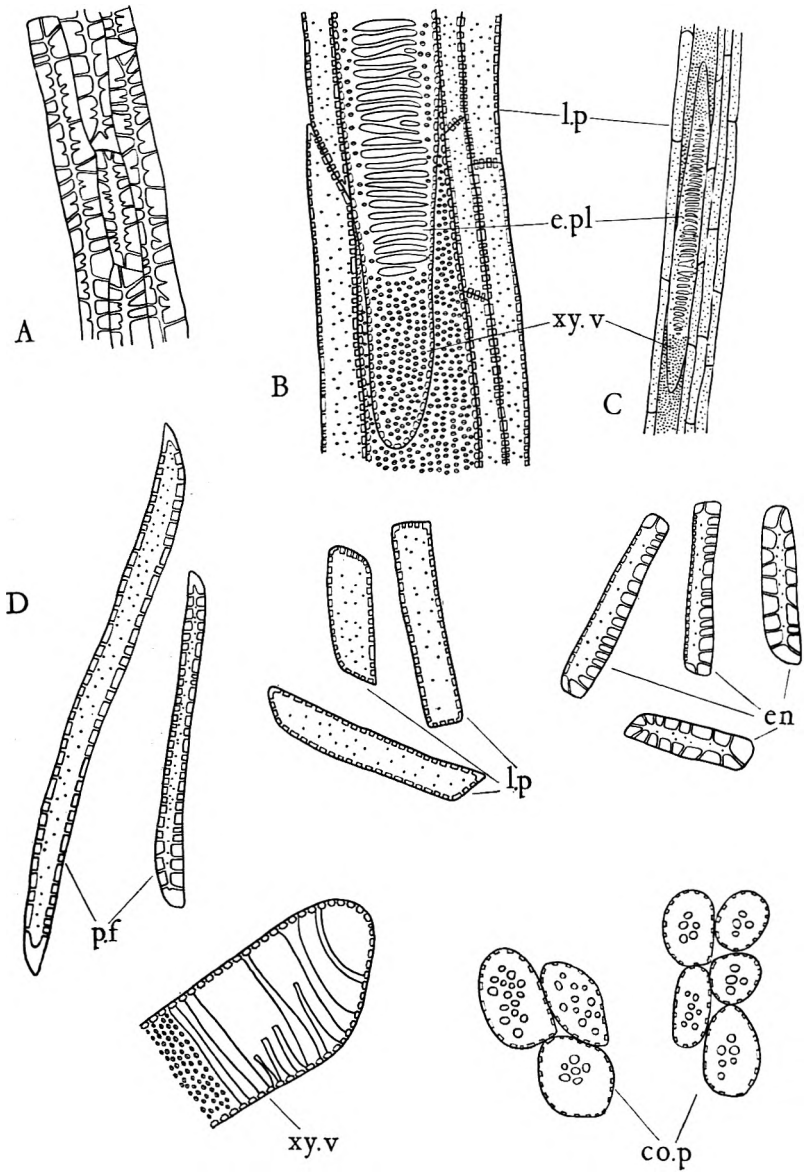

FIG. 6. Root of *Dioscorea belizensis* Lundell. A, surface view of endodermis. B, longitudinal section of xylem. C, longitudinal section of xylem showing entire end plate. D, lignified cells isolated by maceration. C × 60, the rest × 210. *co.p.*, cortical parenchyma; *en.*, endodermis; *e.pl.*, end plate; *l.p.*, lignified parenchyma; *p.f.*, pericyclic fibres; *xy.v.*, xylem vessel.

strands (Fig. 4, C). The cells are polygonal in transverse section, and are usually much elongated longitudinally (Figs. 4, E, 5, B and 6, D). The pericyclic cells measure: T and R = 9–**12**–**21**–30 μ and L = 39 to at least 1,200 μ; they have thick, strongly lignified, simple-pitted walls, the lumen being occasionally very narrow.

Between the xylem strands and enclosed by the pericyclic fibres are the phloem groups (Fig. 4, C). The individual phloem areas are small and consist of a large sieve tube of diameter from 15 to 36 μ, to the outside of which is a group of sieve-tubes of very small diameter; the cells are longitudinally elongated and have relatively thin walls (Figs. 4, C and 5, C).

The xylem is composed of nine to fifteen radiating strands each of which has from two to four vessels, which in transverse section appear circular or slightly oval (Fig. 4, C and E). The vessels fall into one of three diameter ranges; the inner vessels are the widest, R = 45–**105**–**144**–159 μ, whilst the protoxylem vessels have the least diameter R = 18–42 μ; the vessels in between have dimensions of R = 33–**42**–**75**–99 μ (Fig. 4, C). The vessels have thick, lignified walls with numerous bordered pits which have extended pit apertures. The vessels taper at both ends and the end plates have large openings which are continuous with similar openings in the end plates of the overlapping vessels (Figs. 5, D and E, and 6, B, C and D).

The pith consists of cells with thick, lignified, yellow walls containing simple pits. Transversely the cells appear oval due to slight radial elongation, but longitudinally they are much elongated (Figs. 4, E, 5, F and 6, D); they closely resemble the cells of the pericycle, although those of the pith usually have a wider cell diameter and a shorter cell length than the pericyclic cells; L = 36–850 μ, R = 10–**21**–**30**–48 μ and T = 10–**14**–**20**–45 μ.

Acknowledgements. We wish to thank Mr. R. Waters, the Conservator of Forests at Belize, British Honduras, for his great help in collecting the samples of *D. belizensis*. One of us (G.B.) thanks the Tropical Products Institute, London, for the Department of Scientific and Industrial Research Studentship which enabled him to carry out this investigation.

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A NOTE ON THE EFFECTS OF HAMYCIN ON THE PERFUSED RAT HEART

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The effects of hamycin, a cardiotonic polyene antibiotic, were studied on the perfused heart of the albino rat, a species resistant to the digitalis glycosides. Perfusion with a concentration of 1×10^{-6} g./ml. induced systolic arrest in 15 to 20 min. This was preceded by auriculo-ventricular block and a decrease in the heart rate. Effects on the amplitude of contraction and coronary flow were however variable. Ouabain. 2×10^{-6} g./ml. and propylene glycol, which was the solvent for hamycin, did not have any significant effects on this preparation.

The digitalis-like activity of hamycin, a new antifungal antibiotic, has been reported by Arora (1962), and Arora and Arora (1963). Its effects on the rat heart, a species resistant to the known digitalis glycosides, are described below.

Experimental and Results

Hearts from albino rats were perfused (Langendorff's preparation) with Ringer-Locke solution (NaCl 0·9, KCl 0·042, $CaCl_2$ 0·024, $NaHCO_3$ 0·03, Glucose 0·05 per cent) at 35°. After a stabilisation period of 30 to 45 min. drug perfusion was started. Fourteen experiments were made with hamycin 1×10^{-6} g./ml., four were made with ouabain 2×10^{-6} g./ml. and four with Ringer-Locke solution containing the same amount of

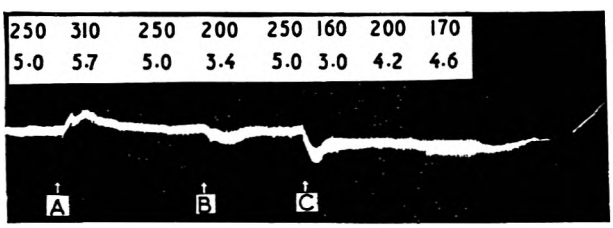

Fig. 1. Perfused rat heart (Langendorff's preparation). Records from above downwards are: heart rate/min.; coronary outflow ml./min. and contractions of the heart. Drugs were injected as follows; A, adrenaline 0·1 μg. B, posterior pituitary extract 0·1 unit; C, start of perfusion with hamycin 1×10^{-6} g./ml.

propylene glycol as in experiments with hamycin. Solutions of hamycin were first made in propylene glycol at 85°, before diluting in physiological solutions. Also, in these experiments, the Ringer-Locke solution used for perfusing the hearts before starting hamycin perfusion contained the same amount of propylene glycol as contained in Ringer-Locke solution with hamycin. Since hamycin induced systolic contracture in 15 to 20 min., experiments with ouabain and propylene glycol were also run for 20 min.

only. Sensitivity of the perfused hearts to adrenaline and posterior pituitary extract was tested in 7 preparations before beginning drug perfusion. Consistent effects on heart rate, coronary flow and tone were obtained with doses of 0·1 μg. of adrenaline and 0·1 unit of posterior pituitary extract, injected into the cannula (Fig. 1). Doses of 0·01 μg. of adrenaline and 0·01 unit of posterior pituitary extract were ineffective.

Hamycin induced complete systolic contracture in 15 to 20 min. in all the 14 experiments but the events preceding this were variable. An initial decrease in the tone occurred in all experiments and was followed by a partial or complete recovery in 7 (Fig. 1). In the other 7 experiments the tone continued to decrease till complete cessation of ventricular activity (Fig. 2). The ventricles then passed gradually into systolic

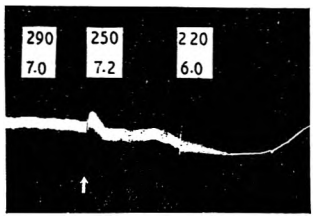

FIG. 2. Perfused rat heart (Langendorff's preparation). Records from above downwards are: heart rate/min.; coronary outflow ml./min. and contractions of the heart. At arrow perfusion with hamycin, 1×10^{-6} g./ml. was started.

contracture. In all but 3 experiments an incomplete auricular-ventricular (a-v) block followed the decrease in tone. The auricles however continued to beat after the ventricles had stopped. An increase in the amplitude of contraction was seen in 9 experiments, occurring either before or after the onset of a-v block; in the remaining 5 experiments both the tone and the amplitude of contraction continued to decrease till the ventricles stopped (Fig. 2). Before the onset of a-v block, a mean decrease of 21·1 per cent $\pm$ 2·19 s.e. was noted in the heart rate but the coronary flow was not consistently effected. Two additional experiments in which a-v block appeared within 1 min. of the onset of perfusion and one more experiment in which the heart rate was not recorded, are not included in these statistics.

Ouabain, 2×10^{-6} g./ml., failed to induce systolic arrest. In 2 of the 4 experiments, a slight increase in the amplitude of contraction and a slight decrease in the heart rate was seen. Coronary flow was not affected significantly.

Control preparations did not show any alteration in the tone, amplitude of contraction, heart rate or coronary flow over the duration of the experiments.

The ability of hamycin to decrease heart rate and induce a-v block and systolic arrest in the rat heart, further confirms its cardiotonic activity. Since the rat heart is resistant to the digitalis glycosides, the interesting possibility of the application of hamycin in digitalis resistant cases would

deserve a consideration if and when a clinical application can be affected.

Acknowledgments. The author thanks the Principal and the Professor of Pharmacology Maulana Azad Medical College, New Delhi, India for facilities; Shri S. L. Kapoor for technical assistance; Dr. B. B. Gokhalay, Poona India for hamycin and Dr. K. K. Chen, Eli Lilly and Co. Indianapolis, U.S.A. for ouabain.

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LETTERS TO THE EDITOR

The Effects of Smooth Muscle Stimulants on the Movement of Calcium-47 in the Guinea-pig Ileum *in vitro*

SIR,—In depolarised strips of rabbit ileum, acetylcholine increases calcium-45 uptake (Robertson, 1950), and Schatzman (1961) has shown that it increases calcium efflux in the guinea-pig taenia coli, and he concluded that during stimulation there was a net calcium uptake. Chujyo and Holland (1962) showed that pilocarpine increased calcium-exchange in a tubular segment of guinea-pig ileum and Durbin and Jenkinson (1961a,b) suggested that the carbachol-induced contracture of the depolarised taenia coli of the guinea-pig was due to a net increase in calcium influx, caused by a drug-induced increase in membrane permeability.

TABLE I

DRUG EFFECTS UPON ^{47}Ca UPTAKE AND RELEASE IN STRIPS OF GUINEA-PIG ILEUM

Drug	Dose (μg./ml.)	Effect on ^{47}Ca release	Effect on ^{47}Ca uptake
Acetylcholine chloride	100	Increased (12) $P < 0.001$	Increased (16) $0.001 < P < 0.01$
Carbachol	6	No change (12) $0.05 < P < 0.10$	Increased (12) $0.02 < P < 0.05$
Histamine acid phosphate	10	Increased (12) $P < 0.001$	No change (12) $0.05 < P < 0.10$
5-Hydroxytryptamine creatinine sulphate..	10	Increased (12) $P < 0.001$	Increased (12) $0.02 < P < 0.05$
Barium chloride	1 mg.	Increased (12) $P < 0.001$	Increased (12) $0.02 < P < 0.05$
Papaverine sulphate	200	Decreased (11) $0.02 < P < 0.05$	No change (12) $0.70 < P < 0.80$
Lysergic acid diethylamide	10	No change (8) $0.20 < P < 0.30$	No change (12) $0.60 < P < 0.70$
Atropine sulphate	10	No change (8) $0.60 < P < 0.70$	No change (12) $0.40 < P < 0.50$
Mepyramine maleate	10	No change (8) $0.90 < P$	No change (12) $0.50 < P < 0.60$
Adrenaline Hydrogen-tartrate	500	No change (12) $0.40 < P < 0.50$	No change (12) $0.50 < P < 0.60$
Noradrenaline bitartrate	500	Increased (12) $0.02 < P < 0.05$	No change (12) $0.20 < P < 0.30$

Figures in parentheses indicate the number of paired strips of guinea-pig ileum used.

We have investigated the effects of some drugs which stimulate smooth muscle, and some of their antagonists upon the influx and efflux of calcium-47 in strips of guinea-pig ileum maintained isotonically in oxygenated Krebs' fluid at 37°. Replicate experiments were made for each dose of drug used, employing not less than eight to twelve paired strips of ileum, one member of each pair serving as control. In the experiments on calcium efflux, the loss of calcium-47 from the tissue during a 1-min. period was estimated; calcium-47 uptake was measured for a period of 10 min. In the former, the total counts released are added to those remaining in the tissue at the end of the experiment, so as to obtain the total initial radioactivity. From this, the percentage of

counts released during exposure to the drug was calculated and compared with those released during exposure to the control solution.

In the influx experiments the counts taken up by the tissue from the drug-containing, radioactive bathing medium were measured, expressed as a percentage and compared with those taken up by a control muscle strip.

In both cases the differences between the drug-treated and control preparations were tested for significance, using Student's '*t*' test. The results are shown in Table I.

Acetylcholine significantly increased calcium-47 release. This confirms the results of Schatzman (1961), who used a lower dose-10 μg./ml. Carbachol did not significantly increase the release at the dose used but histamine, 5-hydroxytryptamine (5-HT) and barium chloride did increase the release as did noradrenaline. Adrenaline, however, had no significant effect, but papaverine sulphate decreased calcium-47 release. Atropine sulphate, mepyramine maleate and lysergic acid diethylamide caused no significant change in release. Eichler, Appel and Staib (1960), using an *in vivo* preparation of rat intestine, showed, however, that 100 μg./ml. of adrenaline increased calcium excretion into the lumen of the intestine.

A significantly increased uptake of calcium-47 was caused by acetylcholine chloride, carbachol, 5-HT and barium chloride. The other compounds tested had no significant effect. The results with acetylcholine and carbachol confirm those of Robertson (1960) and Durbin and Jenkinson (1961a,b).

It is possible that compounds which stimulate the guinea-pig ileum increase both the release and uptake of calcium ions and therefore increase their mobility. Of particular interest is the finding that papaverine sulphate, which causes relaxation of smooth muscle, decreases the release of calcium-47. It has also been shown that papaverine sulphate (0·2 mg./ml.) depressed both the release ($P < 0{\cdot}001$) and uptake ($P < 0{\cdot}001$) of potassium-42 in similar preparations.

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April 5, 1963

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Orally Effective Hypoglycaemic Principles from *Coccinia indica* Wight and Arn

SIR,—*Coccinia indica* is used as a household remedy for diabetes mellitus in the states of Bengal and Bihar in India. Fresh juice from the tuberous roots, stem and leaves is given either by itself or with certain metallic preparations in early cases of diabetes (Nadkarni, 1954).

The hypoglycaemic effect of the root-extract of this plant in alloxan diabetic rabbits was reported by Mukerji (1953), but according to Chopra and Bose (1925a,b), the fresh juice of the plant had no effect on the blood sugar of diabetic patients and fasting rabbits.

Work in this laboratory has shown that the ethanolic and aqueous extracts of sun dried and defatted root powder of *C. indica* contain an orally-effective hypoglycaemic principle. The present communication describes the biological assay, the dose effect relation, and the effect on alloxan diabetic rabbits, of this active principle.

The results obtained according to the methods described earlier for the biological assay (1961a) are given in Table I. The dose-effect relation (1961b) was found to be linear when log dose were plotted against the effect as a per cent of tolbutamide activity. The effects on alloxan diabetes (1962) are given in Table II.

TABLE I

BIOLOGICAL ASSAY OF ORALLY EFFECTIVE HYPOGLYCAEMIC FRACTIONS FROM *Coccinia indica*

Drug tested, g./kg.	Blood sugar response. Mean values (mg./100 ml.) for six rabbits in each group		Mean reduction in blood sugar per cent	Hypoglycaemic potency as per cent of tolbutamide	Significance
	Initial	4 hr. Pool			
Tolbutamide, 0·25 ..	(*a*) 112·9 ± 5·4 (*b*) 118·2 ± 6·1	84·7 ± 6·1 82·1 ± 4·3	25 30·5	100	$t = 2·75$ $P > 0·01$
Alcoholic extract of the root powder of *C. indica* left after ether extraction, 1·25 ..	(*a*) 120·1 ± 5·4 (*b*) 122·5 ± 5·3	101·85 ± 6·8 101·1 ± 5·9	15·2 17·5	58·9	$t = 1·23$ $P < 0·3$
Aqueous extract of the residue left from above, 1·25	(*a*) 115·6 ± 5·7 (*b*) 112·3 ± 6·3	104·5 ± 5·5 104·4 ± 6·2	9·6 7·0	29·9	$t = 1·6$ $P < 0·2$
Control (distilled water)	121·2 ± 5·4	118·2 ± 5·9	2·5	—	—

TABLE II

HYPOGLYCAEMIC ACTION OF *C. indica* COMPARED WITH TOLBUTAMIDE ON THE BLOOD SUGAR OF ALLOXAN DIABETIC RABBITS

Drug, g./kg.	Mean blood sugar values for six rabbits in each group (mg./100 ml.)		Maximum fall in fasting blood sugar per cent	Significance
	Initial	Min. in 4 hr.		
Tolbutamide 0·25	(*a*) 202·3 ± 7·2 (*b*) 265·2 ± 6·8	161·6 ± 7·4 201·0 ± 6·4	20·1 24·2	$t = 2·31$ $P < 0·05$
C. indica extract (ethanolic) 1·25	(*a*) 235·2 ± 6·8 (*b*) 190·3 ± 7·2	220·2 ± 8·2 174·7 ± 7·6	6·4 8·2	$t = 1·402$ $P < 0·2$
Control (distilled water) ..	190·4 ± 7·6	186·3 ± 7·1	2·20	—

These results indicate that the roots of *C. indica* contain an orally-effective hypoglycaemic principle comparable to tolbutamide.

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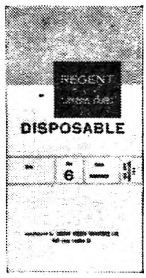

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