BRITISH PHARMACEUTICAL CONFERENCE

NINETY-SEVENTH ANNUAL MEETING, NEWCASTLE UPON TYNE, 1960

REPORT OF PROCEEDINGS

OFFICERS:

President:

T. Reid, M.P.S., Haslemere

Chairman:

W. H. Linnell, D.Sc., Ph.D., F.P.S., F.R.I.C., London

Vice-Chairmen:

R. R. Bennett, B.Sc., F.P.S., F.R.I.C., Eastbourne.
Harold Deane, B.Sc., F.P.S., F.R.I.C., Sudbury.
H. Humphreys Jones, F.P.S., F.R.I.C., Liverpool.
T. E. Wallis, D.Sc., F.P.S., F.R.I.C., F.L.S., London.
H. Brindle, M.Sc., F.P.S., F.R.I.C., Altrincham.
Norman Evers, B.Sc., Ph.D., F.R.I.C., Ware.
A. D. Powell, M.P.S., F.R.I.C., Nottingham.
H. Berry, B.Sc., Dip.Bact. (Lond.), F.P.S., F.R.I.C., Eastbourne.
H. B. Mackie, B.Pharm., F.P.S., Brighton.
G. R. Boyes, L.M.S.S.A., B.Sc., F.P.S., F.R.I.C., London.
H. Davis, C.B.E., B.Sc., Ph.D., F.P.S., F.R.I.C., London.
J. P. Todd, Ph.D., F.P.S., F.R.I.C., Glasgow.
K. Bullock, M.Sc., Ph.D., F.P.S., F.R.I.C., Manchester.
Frank Hartley, B.Sc., Ph.D., F.P.S., F.R.I.C., London.
G. E. Foster, B.Sc., Ph.D., F.R.I.C., Dartford.
H. Treves Brown, B.Sc., F.P.S., London.

Honorary Treasurer:

H. G. Rolfe, B.Sc., F.P.S., F.R.I.C., London.

Honorary General Secretaries:

E. F. Hersant, B.Pharm., Ph.D., F.P.S., F.R.I.C., London.
D. Train, M.C., Ph.D., F.P.S., F.R.I.C., A.M.I.Chem.E.

Other Members of the Executive Committee:

The Chairman of the Executive of the Scottish Department of the Pharmaceutical Society of Great Britain (*ex officio*).
The President of the Pharmaceutical Society of Ireland (*ex officio*).
The President of the Pharmaceutical Society of Northern Ireland (*ex officio*).
The Editor of the *Journal of Pharmacy and Pharmacology* (*ex officio*).
The Chairman and Honorary Secretary of the Local Committee (*ex officio*).

D. C. M. Adamson, F.R.I.C., Greenford.
A. W. Bull, B.Sc., B.Pharm., F.P.S., F.R.I.C., Nottingham.
J. G. Dare, Ph.D., F.P.S., Leeds.
*D. W. Hudson, M.P.S., Hove.
*G. H. Hughes, M.P.S., Colwyn Bay.
*H. S. Grainger, F.P.S., London.
G. F. Somers, B.Sc., Ph.D., F.P.S., M.I. Biol., Liverpool.
J. B. Stenlake, D.Sc., Ph.D., F.P.S., F.R.I.C., Glasgow.
W. T. Wing, F.P.S., D.B.A., Newcastle.

* Members nominated by the Council of the Pharmaceutical Society of Great Britain.

REPORT OF PROCEEDINGS

PROCEEDINGS OF CONFERENCE

NEWCASTLE UPON TYNE 1960

THE OPENING SESSION

The opening session of the Conference was held in the City Hall in Newcastle upon Tyne on Monday, September 5, with Mr. T. Reid, President of the Conference (President of the Pharmaceutical Society), in the Chair. On the platform were the Chairman of the Conference (Professor W. H. Linnell), the Lord Mayor of Newcastle upon Tyne (Mrs. G. Robson), the Chairman and Secretary of the Local Committee (Mr. T. D. Clarke and Mr. A. McGuckin), the Honorary General Secretaries together with members of the Conference Executive Committee.

The President introduced the Lord Mayor of Newcastle, who welcomed the Conference to the City. The President thanked the Lord Mayor on behalf of the Conference for her welcome.

The President then handed over further conduct of the Conference to the Chairman (Professor W. H. Linnell), who delivered his address entitled "Education and Research," which is printed in the *Pharmaceutical Journal*, 1960, **185**, pages 227–228.

On the proposition of Dr. A. H. Beckett, seconded by Mr. A. R. G. Chamings, the Conference accorded a vote of thanks to the Chairman for his address.

CIVIC RECEPTION

On the evening of Monday, September 5, a Civic Reception was given at the Old Assembly Rooms. The guests were received by the Lord Mayor and the Lady Mayoress (Mrs. E. Murray). A dance was held after the reception.

THE SCIENCE SESSIONS

Meetings were held on Monday, Tuesday, Wednesday and Friday, September 5, 6, 7, 9 at King's College, the Chairman and immediate Past Chairman presiding. During the sessions the following 33 papers were communicated:—

1. Alkaloids of *Voacanga schweinfurthii* Stapf. Part I. Voacamine and Vobtusine. By F. Fish, B.Pharm., Ph.D., F.P.S., F. Newcombe, B.Sc., A.R.C.S.T., M.P.S. and J. Poisson, Pharmacien, D. ès Sc.
2. Vegetable Purgatives Containing Anthracene Derivatives. Part XI. Further Work on the Aloin-like Substance of *Rhamnus purshiana* DC. By J. W. Fairbairn, B.Sc., Ph.D., F.P.S., F.L.S., F.R.I.C. and S. Simic, B.Pharm.
3. Anatomical Studies in the Genus Digitalis. Part II. The Anatomy of the Inflorescence of *D. lanata L.* By P. S. Cowley, B.Pharm., M.P.S., and J. M. Rowson, M.Sc., Ph.D., F.P.S., F.L.S.
4. Studies in the Genus Digitalis. Part VI. Variations in Glycosidal Content of British Clones of *Digitalis purpurea.* By J. M. Rowson, M.Sc., Ph.D., F.P.S., F.L.S.
5. Studies in the Genus Digitalis. Part VII. Variations in Glycosidal Content Within Clones of *Digitalis purpurea.* By J. M. Rowson, M.Sc., Ph.D., F.P.S., F.L.S.
6. The Strength of Compressed Tablets. Part I. The Measurement of Tablet Strength and its Relation to Compression Forces. By E. Shotton, B.Sc., Ph.D., F.P.S., F.R.I.C. and D. Ganderton, B.Pharm.
7. The Strength of Compressed Tablets. Part II. The Bonding of Granules during Compression. By E. Shotton, B.Sc., Ph.D., F.P.S., F.R.I.C. and D. Ganderton, B.Pharm.
8. The Use of Laminar Lubricants in Compaction Processs. By D. Train, M.C., B.Pharm., B.Sc. (Eng.), Ph.D.,[A.C.G.I., D.I.C., F.P.S., F.R.I.C., A.M.I.Chem.E. and J. A. Hersey, B.Pharm., Ph.D.
9. The Emulsifying Properties of Gum Acacia. By E. Shotton, B.Sc., Ph.D., F.P.S., F.R.I.C. and K. Wibberley, B.Pharm., M.P.S., A.R.I.C.
10. Rheology of Acacia-stabilised Emulsions. By E. Shotton, B.Sc., Ph.D., F.P.S., F.R.I.C. and R. F. White, B.Pharm.
11. A Note on the Stability of Solutions of Phenylephrine. By G. B. West, B.Pharm., D.Sc., Ph.D., F.P.S. and T. D. Whittet, B.Sc., Ph.D., F.P.S., F.R.I.C., D.B.A.
12. Preliminary Studies of the Heat Resistance of Bacterial Spores on Paper Carriers. By A. M. Cook, B.Pharm., Ph.D., Dip.Bact., F.P.S., F.R.I.C. and M. R. W. Brown, M.Sc., M.P.S.

13. Mould Spore Suspensions and Powders for Use in Fungicidal Kinetic Studies. Part I. Preliminary Experiments with *Rhizopus nigricans* and *Penicillium digitatum*. By M. R. W. Brown, M.Sc., M.P.S. and Kenneth Bullock, M.Sc., Ph.D., F.P.S., F.R.I.C.
14. Mould Spore Suspensions and Powders for Use in Fungicidal Kinetic Studies. Part II. Preparations using *Penicillium spinulosum*. By H. N. Gerrard, M.Sc., B.Comm., F.P.S., Ann V. Harkiss, M.Sc. and Kenneth Bullock, M.Sc., Ph.D., F.P.S., F.R.I.C.
15. The Effect of Pipetting on the Concentration of Homogeneous Spore Suspensions. By H. N. Gerrard, M.Sc., B.Comm., F.P.S. and G. S. Porter, F.P.S.
16. Neuromuscular Blocking Agents. Part VII. Linear Polyonium Ethers. By D. Edwards, B.Sc., F.P.S., F.R.I.C., J. J. Lewis, M.Sc., F.P.S., D. E. McPhail, B.Sc., M.P.S., T. C. Muir, B.Sc., M.P.S. and J. B. Stenlake, D.Sc., Ph.D., F.P.S., F.R.I.C.
17. The Effect of Thalidomide in Experimental Gastric Ulcers. By K. Martindale, M.B., Ch.B., G. F. Somers, B.Sc., Ph.D., F.P.S., M.I.Biol. and C. W. M. Wilson, B.Sc., Ph.D., M.D.
18. An Enzymatic Method for the Determination of Prednisolone Phosphate in Pharmaceutical Preparations. By P. F. G. Boon, B.Sc., A.R.I.C.
19. The Precision of Some Procedures in Pharmaceutical Analysis. Part II. Titrations. By A. R. Rogers, B.Sc., B.Pharm., F.P.S., A.R.I.C.
20. Physico-Chemical Studies of (1-Methyl-2-pyrrolidyl)methyl Benzilate Methyl Methosulphate. Part I. The Determination of (1-Methyl-2-pyrrolidyl)methyl Benzilate Methyl Methosulphate in the Presence of its Breakdown Products. By D. O. Singleton, B.Sc. and (Miss) G. M. Wells, B.Sc., A.P.I.
21. Separation and Estimation of Purine and Pyrimidine Bases from a Heated Suspension of *Micrococcus flavus*. By K. W. Gerritsma, D.Sc. and H. P. Levius, M.Sc., D.Sc., F.P.S.
22. An Investigation into the Effectiveness of Various Antoxidants on the Preservation of Fresh Groundnut Oil (Food Grade) B.P. By G. A. Birchall, M.P.S. and R. I. Felix, F.P.S., A.R.I.C.
23. An Examination of the Decomposition of Dextrose Solution during Sterilisation. By W. T. Wing, F.P.S., D.B.A.
24. A Shorter Sterilising Cycle for Solutions Heated in an Autoclave. By G. R. Wilkinson, F.P.S., F. G. Peacock, and E. L. Robins, B.Pharm., A.R.I.C.
25. Some Factors Involved in Multiple Spot Formation in the Paper Chromatography of Sympathomimetic Amines in the Presence of Acids. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C., M. A. Beaven, B.Pharm. and Ann E. Robinson, B.Pharm., Ph.D., A.R.I.C.
26. The Assay of Stilboestrol by the Isotope Dilution Technique. By R. Fleming, B.Pharm., Ph.D., A.R.I.C.
27. The Determination of *n*-Values for some Aminoacridines by Controlled Potential Coulometric Reduction. By (Miss) F. P. Wilson, M.P.S., C. G. Butler, B.Pharm., M.P.S., F.R.I.C., P. H. B. Ingle, F.P.S. and H. Taylor, B.Sc., Ph.D., F.P.S., F.R.I.C.
28. The Determination of the Relative Configuration of Morphine, Levorphanol and *Laevo*-Phenazocine by Stereoselective Adsorbents. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C. and Patricia Anderson, B.Sc. (Pharm.), F.P.S.
29. Preparation of a ^{35}S Labelled Trimeprazine Tartrate Sustained Action Product for its Evaluation in Man. By Earl Rosen, B.S. and Joseph V. Swintosky, B.S., Ph.D.
30. The Assimilation and Elimination of Iron Administered Orally to the Dog as Ferrous Isoascorbate and Ferrous Ammonium Sulphate. By H. D. C. Rapson, B.Sc., Ph.D., D.I.C., A.R.I.C., A. A. Lewis, B.Sc., M.D., M.B., B.S., M.R.C.S. (Eng.), M.R.C.P., and Jean M. Coops, B.Sc.
31. Phosphatide Membranes. By L. Saunders, D.Sc., Ph.D., F.R.I.C.
32. Detection of Lysolecithin in a Sample of Egg Lecithin. By L. Saunders, D.Sc., Ph.D., F.R.I.C. and J. Perrin, B.Pharm., M.P.S.
33. The Size, Shape and Hydration of Cetomacrogol 1000 Micelles. By P. H. Elworthy, B.Pharm., Ph.D.

The papers are printed in full with reports of discussions in the *Journal of Pharmacy and Pharmacology*, 1960, **12**, Supplement, pages 41 *T*– 266 *T*.

THE SYMPOSIUM SESSION

A symposium on "Chemical Disinfection" was held on Thursday, September 8. The Chairman presided. The introductory papers were by Drs. S. E. Jacobs,

A. M. Cook and J. G. Davis. Dr. Davis's paper was presented by Mr. A. H. Walters, who added a short contribution on disinfection in hospitals. The meeting is reported in the *Journal of Pharmacy and Pharmacology*, 1960, **12**, Supplement, pages 9 *T*–40 *T*.

PROFESSIONAL SESSIONS

With the President of the Conference, Mr. T. Reid, in the chair, professional sessions were held on the mornings of Tuesday, September 6, when Dr. K. R. Capper read an introductory paper to the subject "The Change to the Metric System in Pharmaceutical Practice," and Friday, September 9, when Mr. C. G. Drummond and Professor A. D. Macdonald read introductory papers to the subject "Professional Responsibilities of the Pharmacist in the Supply of Medicinal Substances." Full reports of the papers and discussions were published in the *Pharmaceutical Journal*, 1960, **185**, pages 263–266; 292–297.

THE CLOSING SESSION

The closing session of the Conference was held on Friday, September 9, in the City Hall, the Chairman presiding.

Vote of Thanks to Local Committee

The Chairman called on Mr. J. G. Coleman to propose a vote of thanks to the Local Committee. This was seconded by Mr. J. B. Grosset. Mr. T. D. Clarke (Chairman of the Local Committee) replied to the vote of thanks. The Chairman then presented to the Newcastle District and Northumberland Branch an inscribed gavel provided by the Bell and Hills Fund. Mr. G. H. M. Graham (Chairman of the Branch) accepted and acknowledged the gift on behalf of the Branch.

Annual Report

Dr. E. F. Hersant presented the Annual Report of the Executive Committee.

Your Executive has pleasure in presenting the ninety-seventh Annual Report.

Membership.—In addition to Members, Honorary Members and Students of the Pharmaceutical Society, together with the Members of the Pharmaceutical Society of Northern Ireland, the Conference includes sixty-six members elected by the Executive who are not members of these Societies.

Obituary.—Your Executive regrets to report the death of B. A. Bull, Chairman of the Conference in 1946 and 1947.

Reports on 1959 Meeting.—The report of the meeting of the Conference at Bournemouth 1959 together with the science papers and discussions were published as a supplement to the eleventh volume of the *Journal of Pharmacy and Pharmacology*. The papers and discussions at the Professional Sessions were published in the *Pharmaceutical Journal*, Series IV, Volume 129.

Conference Papers 1960.—Thirty-five research papers were submitted. Twenty-seven full papers and six short communications were accepted for presentation. The Executive thanks the authors of these papers and also the authors of the papers presented to the Symposium and the Professional Sessions for their contributions. The Executive is grateful to the Editor of the *Journal of Pharmacy and Pharmacology* and to the Editor of the *Pharmaceutical Journal* for making galley proofs of the papers available before this meeting.

Journal of Pharmacy and Pharmacology.—The Executive has been represented on the Editorial Board by the Chairman (Professor W. H. Linnell), the immediate Past-Chairman (Mr. H. Treves Brown) and the Senior Honorary General Secretary.

Future Meetings.—An invitation will be presented at this meeting for the Conference to meet in Portsmouth during the week commencing September 18, 1961. In addition to the invitations provisionally accepted last year, your Executive has also provisionally accepted an invitation to visit Edinburgh in 1964. Other Branches of the Society have made preliminary enquiries regarding the possibility of entertaining the Conference in future years and the Executive is grateful for all these offers of hospitality.

Officers and Executive of the Conference.—Your Executive has nominated the following Officers and Members of the Executive for 1960–1961:—

Chairman: D. C. Garratt, *Vice-Chairmen:* R. R. Bennett, H. Deane, H. Humphreys Jones, T. E. Wallis, H. Brindle, Norman Evers, A. D. Powell, H. Berry, H. B. Mackie, G. R. Boyes, H. Davis, J. P. Todd, K. Bullock, Frank Hartley, G. E. Foster, H. Treves Brown and W. H. Linnell. *Honorary*

Treasurer: H. G. Rolfe, *Honorary General Secretaries:* E. F. Hersant and D. Train. Other members of the Executive: K. R. Capper, G. F. Somers, J. B. Stenlake, R. E. Stuckey, G. Sykes and W. T. Wing.

The above persons together with the President of the Conference (the President of the Pharmaceutical Society of Great Britain *ex officio*), the three persons nominated by the Council of the Pharmaceutical Society of Great Britain, namely the persons for the time being holding the office of Vice-President, immediate past President and Chairman of the Organisation Committee, together with the following *ex officio*: The Chairman of the Executive of the Scottish Department, the President of the Pharmaceutical Society of Ireland, the President of the Pharmaceutical Society of Northern Ireland, the Editor of the *Journal of Pharmacy and Pharmacology*, the Chairman of the Local Committee and the Honorary Local Secretary, will form the Executive for 1960–1961.

ACKNOWLEDGEMENTS.—The Executive wishes to record thanks to the Chairman, Officers and Members of the Newcastle upon Tyne Local Committee for their work in making the local arrangements, and to the Council of the Pharmaceutical Society of Great Britain for the continued provision of secretarial and other facilities.

Dr. J. M. Rowson proposed the acceptance of the report and the election of officers of the Conference for the ensuing year. Miss M. A. Burr seconded.

Dr. D. C. Garrett thanked the Conference on behalf of the newly-elected officers.

TREASURER'S REPORT

Mr. H. G. Rolfe presented and proposed the adoption of the following Report and Statement of Accounts for the year 1960:—

The accounts for the year ended December 31, 1959, show that the stock of replicas of the Chairman's Badge has been replenished by the purchase of six new badges and these are included at cost (£44 2*s.* 0*d.*).

The Local Committee Fund of £250 having been repaid by the Bournemouth Local Committee, was loaned to the Newcastle upon Tyne Local Committee in October, 1959, in respect of the 1960 Conference.

The subscriptions from elected members, including a composition fee from the Pharmaceutical Society of Northern Ireland, amounted to £154 10*s.* 0*d.* and were credited to the account of the *Journal of Pharmacy and Pharmacology*.

H. G. ROLFE,
Honorary Treasurer.

BRITISH PHARMACEUTICAL CONFERENCE ACCOUNT

INCOME AND EXPENDITURE ACCOUNT, 1959

Expenditure	£	*s.*	*d.*	*Income*	£	*s.*	*d.*
Gavel—memento to Bournemouth and District Branch	8	16	3	Interest on 2½% Consols	40	5	0
Replica of Chairman's Badge, engraving, etc.	7	7	6	Interest on 3% Savings Bonds	6	0	0
Check book		4	0	Interest on 3% Exchequer Stock	15	0	0
Plinth and engraving Edmund White Golf Trophy	5	0	0	Interest on P.O. Savings Bank Account		5	1
Income Tax for the year	24	16	1	Interest on Bank Deposit Account		8	3
Secretaries' expenses	39	3	8	Donation from Pharmaceutical Society of Northern Ireland	25	0	0
Surplus carried to Accumulated Fund	26	10	10	Donation from Pharmaceutical Society of Ireland	25	0	0
	£111	18	4		£111	18	4

BALANCE SHEET AT DECEMBER 31, 1959

Liabilities	£	*s.*	*d.*	*Assets*	£	*s.*	*d.*
Accumulated Fund, as at 31.12.58:—	1,982	4	4	Investments at cost (*a*) £1,610 2½% Consols (Donation by the late Alderman Clayton of Birmingham)	1,250	0	0
Add: Surplus 1959	26	10	10	(*b*) £200 3% Savings Bonds 1960–70	200	0	0
	2,008	15	2	(*c*) £500 3% Exchequer Stock 1962–63 (Total Market value at December 31, 1959: £1,453)	473	4	10
Income Tax due	9	3	8	Stock of Replicas (6) of Chairman's Badge	44	2	0
Local Committee Fund:— Donation from London Committee, 1953	250	0	0	Loan to Newcastle Local Committee	250	0	0
				Post Office Savings Bank Account	6	3	1
				Cash at Westminster Bank	44	8	11
	£2,267	18	10		£2,267	18	10

Audited and found correct
T. HESELTINE
J. C. HANBURY

May 31, 1960

The President seconded, and the Report was adopted.

REPORT OF PROCEEDINGS

BRITISH PHARMACEUTICAL CONFERENCE

INAUGURAL MEETING HELD AT NEWCASTLE UPON TYNE IN 1863

Years	*Places of Meeting*	*Presidents*	*Local Secretaries*
1864	BATH	HENRY DEANE, F.L.S.	J. C. POOLEY.
1865	BIRMINGHAM ..	HENRY DEANE, F.L.S.	W. SOUTHALL, JUN.
1866	NOTTINGHAM ..	PROF. BENTLEY, F.L.S.	J. H. ATHERTON, F.C.S.
1867	DUNDEE ..	PROF. BENTLEY, F.L.S.	J. HODGE.
1868	NORWICH ..	DANIEL HANBURY, F.R.S.	F. SUTTON, F.C.S.
1869	EXETER ..	DANIEL HANBURY, F.R.S.	M. HUSBAND.
1870	LIVERPOOL ..	W. W. STODDART, F.C.S.	E. DAVIES, F.C.S. J. DUTTON (Birkenhead).
1871	EDINBURGH ..	W. W. STODDART, F.C.S.	J. MACKAY, F.C.S.
1872	BRIGHTON ..	H. B. BRADY, F.R.S.	T. GLAISYER.
1873	BRADFORD ..	H. B. BRADY, F.R.S.	R. PARKINSON, PH.D.
1874	LONDON ..	THOS. B. GROVES, F.C.S.	M. CARTEIGHE, F.C.S.
1875	BRISTOL ..	THOS. B. GROVES, F.C.S.	J. PITMAN.
1876	GLASGOW ..	PROF. REDWOOD, F.C.S.	A. KINNINMONT.
1877	PLYMOUTH ..	PROF. REDWOOD, F.C.S.	R. J. CLARK.
1878	DUBLIN ..	G. F. SCHACHT, F.C.S.	W. HAYES.
1879	SHEFFIELD ..	G. F. SCHACHT, F.C.S.	H. MALEHAM.
1880	SWANSEA ..	W. SOUTHALL, F.L.S.	J. HUGHES.
1881	YORK	R. REYNOLDS, F.C.S.	J. OWRAY.
1882	SOUTHAMPTON	PROF. ATTFIELD, F.R.S.	O. R. DAWSON.
1883	SOUTHPORT ..	PROF. ATTFIELD, F.R.S.	WM. ASHTON.
1884	HASTINGS ..	J. WILLIAMS, F.C.S.	F. ROSSITER.
1885	ABERDEEN ..	J. B. STEPHENSON.	A. STRACHAN.
1886	BIRMINGHAM ..	T. GREENISH, F.C.S.	CHAS. THOMPSON.
1887	MANCHESTER ..	S. R. ATKINS, J.P.	F. B. BENGER, F.C.S.
1888	BATH	F. B. BENGER, F.C.S.	H. HUTTON.
1889	NEWCASTLE UPON TYNE	C. UMNEY, F.I.C., F.C.S.	T. M. CLAGUE.
1890	LEEDS	C. UMNEY, F.I.C., F.C.S.	F. W. BRANSON, F.C.S.
1891	CARDIFF ..	W. MARTINDALE, F.C.S.	ALFRED COLEMAN.
1892	EDINBURGH ..	E. C. C. STANFORD, F.C.S.	PETER BOA.
1893	NOTTINGHAM ..	OCTAVIUS CORDER	C. A. BOLTON.
1894	OXFORD ..	N. H. MARTIN, F.L.S., F.R.M.S.	H. MATTHEWS.
1895	BOURNEMOUTH	N. H. MARTIN, F.L.S., F.R.M.S.	STEWART HARDWICK.
1896	LIVERPOOL ..	W. MARTINDALE, F.C.S.	T. H. WARDLEWORTH. H. O. DUTTON (Birkenhead).
1897	GLASGOW ..	DR. C. SYMES.	J. A. RUSSELL.
1898	BELFAST ..	DR. C. SYMES	R. W. McKNIGHT. W. J. RANKIN
1899	PLYMOUTH ..	J. C. C. PAYNE, J.P.	J. DAVY TURNEY.
1900	LONDON ..	E. M. HOLMES, F.L.S.	W. WARREN. HERBERT CRACKNELL.
1901	DUBLIN ..	G. C. DRUCE, M.A., F.L.S.	J. I. BERNARD.
1902	DUNDEE ..	G. C. DRUCE, M.A., F.L.S.	W. CUMMINGS.
1903	BRISTOL ..	T. H. W. IDRIS, M.P., F.C.S.	H. E. BOORNE.
1904	SHEFFIELD ..	T. H. W. IDRIS, M.P., F.C.S.	H. ANTCLIFFE.
1905	BRIGHTON ..	W. A. H. NAYLOR, F.I.C., F.C.S.	W. W. SAVAGE. C. G. YATES.
1906	BIRMINGHAM ..	W. A. H. NAYLOR, F.I.C., F.C.S.	C. THOMPSON.
1907	MANCHESTER ..	THOS. TYRER, F.I.C., F.C.S.	W. KIRBY.
1908	ABERDEEN ..	ROBT. WRIGHT, F.C.S.	W. F. HAY.
1909	NEWCASTLE UPON TYNE	J. G. TOCHER, B.Sc., F.R.I.C.	T. M. CLAGUE. H. W. NOBLE.
1910	CAMBRIDGE ..	FRANCIS RANSOM, F.C.S.	A. A. DECK. T. J. MALLETT.
1911	PORTSMOUTH ..	W. F. WELLS.	T. O. BARLOW. T. POSTLETHWAIT.
1912	EDINBURGH ..	SIR EDWARD EVANS, J.P.	THOS. STEPHENSON.
1913	LONDON ..	JOHN C. UMNEY, F.C.S.	W. J. UGLOW WOOLCOCK.
1914	CHESTER ..	E. H. FARR, F.C.S.	R. CECIL OWEN, B.Sc.
1915	LONDON ..	E. SAVILLE PECK, M.A.	———
1916	LONDON ..	DAVID HOOPER, LL.D., F.R.I.C.	———
1917	LONDON ..	CHAS. ALEX. HILL, B.Sc., F.R.I.C.	———
1918	LONDON ..	CHAS. ALEX. HILL, B.Sc., F.R.I.C.	———
1919	LONDON ..	W. KIRBY, M.Sc., F.C.S.	———
1920	LIVERPOOL ..	CHAS. ALEX. HILL, B.Sc., F.R.I.C.	H. HUMPHREYS JONES F.R.I.C.
1921	SCARBOROUGH	E. SAVILLE PECK, M.A.	E. R. CROSS.
1922	NOTTINGHAM ..	PROF. H. G. GREENISH, D. ès. Sc., F.I.C.	E. C. CARR.

Years	*Places of Meeting*	*Chairmen*	*Local Secretaries*
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1924	BATH	EDMUND WHITE, B.Sc., F.I.C.	P. J. THOMPSON. W. H. HALLETT.
1925	GLASGOW ..	EDMUND WHITE, B.Sc., F.I.C.	P. M. DUFF.
1926	LEICESTER ..	D. LLOYD HOWARD, J.P.	J. BARKER.
1927	BRIGHTON ..	D. LLOYD HOWARD, J.P.	F. W. BURGESS.
1928	CHELTENHAM ..	R. R. BENNETT, B.Sc., F.R.I.C.	P. JAMES.
1929	DUBLIN ..	R. R. BENNETT, B.Sc., F.R.I.C.	V. E. HANNA.

Years	*Places of Meeting*	*Chairmen*	*Local Secretaries*
1930	CARDIFF ..	J. T. HUMPHREY	J. MURRAY.
1931	MANCHESTER ..	J. H. FRANKLIN	R. G. EDWARDS.
1932	ABERDEEN ..	H. SKINNER	H. M. DUGAN.
1933	LONDON ..	C. H. HAMPSHIRE, C.M.G., M.B., B.S., B.Sc., F.R.I.C.	H. N. LINSTEAD.
1934	LEEDS	C. H. HAMPSHIRE, C.M.G., M.B., B.S., B.Sc., F.R.I.C.	G. C. CRUMMACK. J. F. SIMON.
1935	BELFAST ..	F. W. CROSSLEY-HOLLAND, L.M.S.S.A.	D. L. KIRKPATRICK.
1936	BOURNEMOUTH	HAROLD DEANE, B.Sc., F.R.I.C.	V. J. SCAMPTON.
1937	LIVERPOOL ..	T. EDWARD LESCHER, O.B.E.	W. E. HUMPHREYS.
1938	EDINBURGH ..	J. RUTHERFORD HILL, O.B.E.	C. G. DRUMMOND.
1939	BIRMINGHAM ..	J. RUTHERFORD HILL, O.B.E.	D. J. RUSTON.
1940	LONDON ..	H. HUMPHREYS JONES, F.R.I.C.	———
1941	LONDON ..	A. R. MELHUISH.	———
1942	LONDON ..	T. E. WALLIS, D.Sc., F.R.I.C., F.L.S.	———
1943	LONDON ..	T. E. WALLIS, D.Sc., F.R.I.C., F.L.S.	———
1944	LONDON ..	H. BRINDLE, B.Sc., F.R.I.C.	———
1945	LONDON ..	H. BRINDLE, B.Sc., F.R.I.C.	———
1946	LONDON ..	B. A. BULL, A.R.I.C.	———
1947	TORQUAY ..	B. A. BULL, A.R.I.C.	T. D. EVANS.
1948	BRIGHTON ..	NORMAN EVERS, B.Sc., PH.D., F.R.I.C.	A. WILSON.
1949	BLACKPOOL ..	NORMAN EVERS, B.Sc., PH.D., F.R.I.C.	R. VARLEY. T. A. DURKIN.
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1953	LONDON ..	G. R. BOYES, L.M.S.S.A., B.Sc., F.R.I.C.	J. M. ROWSON.
1954	OXFORD ..	H. DAVIS, C.B.E., B.Sc., PH.D., F.R.I.C.	T. R. HARDY.
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1956	DUBLIN ..	K. BULLOCK, M.Sc., PH.D., F.R.I.C.	D. J. KENNELLY.
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1958	LLANDUDNO ..	G. E. FOSTER, B.Sc., PH.D., F.R.I.C.	M. H. THOMAS.
1959	BOURNEMOUTH	H. TREVES BROWN, B.Sc., F.P.S.	D. F. SMITH.
1960	NEWCASTLE UPON TYNE	W. H. LINNELL, D.Sc., PH.D., F.P.S., F.R.I.C.	A. McGUCKIN.

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Closing Session (continued)

PLACE OF MEETING FOR 1961

Mr. J. C. Bloomfield on behalf of the Portsmouth and District Branch, extended an invitation to hold the Conference in Portsmouth in 1961.

Mr. J. H. Oakley proposed that the invitation be accepted, and Mr. F. R. C. Bateson seconded. The vote was put to the meeting and unanimously carried.

VOTE OF THANKS TO CHAIRMAN

Mr. R. L. Stephens proposed a vote of thanks to the Chairman.

Mr. D. Nedeljkovic seconded. The vote was put to the meeting by the President and carried with acclamation.

Professor Linnell briefly responded.

REPORT OF PROCEEDINGS

BRITISH PHARMACEUTICAL CONFERENCE

CONSTITUTION AND RULES

1. The British Pharmaceutical Conference shall meet annually for the discussion of subjects relative to the science of pharmacy, for the promotion of friendly reunion amongst pharmacists and those interested in pharmacy, and generally for the furtherance of the objects sought to be obtained under the Royal Charter of Incorporation granted to the Pharmaceutical Society of Great Britain and the several Pharmacy Acts.

2. The Conference shall consist of:—

(*a*) members honorary members and student-associates of the Pharmaceutical Society of Great Britain;

(*b*) members of the other Pharmaceutical Societies within the British Commonwealth of Nations which desire to be associated with the work of the Conference and have made an agreement with the British Pharmaceutical Conference whereby an annual subscription shall be paid by their Society in lieu of individual subscriptions from members; and

(*c*) persons at home and abroad interested in the advancement of Pharmacy who, not being qualified for membership of the Pharmaceutical Society of Great Britain or one of the other Societies associated with the work of the Conference, have been nominated in writing by a member of the Conference, and elected by the Executive.

3. The Officers of the Conference, who shall collectively constitute the Executive Committee, shall be:—A Chairman, Vice-Chairmen, one Honorary Treasurer, two Honorary General Secretaries, together with three members of the Council of the Pharmaceutical Society of Great Britain, and six other members of the Conference. Of the six other members nominated annually by the outgoing Executive the two members who have had the longest period of continuous service shall be ineligible for re-nomination for one year. The President of the Pharmaceutical Society of Great Britain shall be *ex officio* a member of the Executive Committee and President of the Conference. The Chairman of the Executive of the Scottish Department of the Pharmaceutical Society of Great Britain, the President of the Pharmaceutical Society of Ireland, the President of the Pharmaceutical Society of Northern Ireland, the President of any other Pharmaceutical Society the members of which are members of the Conference, the Editor of the *Journal of Pharmacy and Pharmacology*, the Chairman of the Local Committee, and the Honorary Local Secretary shall be *ex officio* members of the Executive Committee.

4. The Officers of the Conference shall be selected at each annual meeting by those present. Nominations for membership of the Executive Committee may be made at the annual meeting by the outgoing Executive and shall include nominations which have been received by the Honorary Secretaries in writing at least twenty-eight days before the annual meeting.

5. The Chairman of the Conference shall give the inaugural address, preside over the meetings for the reading of scientific papers and take the chair at the meetings of the Executive Committee.

6. Members, honorary members and student-associates of the Pharmaceutical Society of Great Britain shall not be required to pay a separate subscription to the British Pharmaceutical Conference; they shall be entitled, on application, to receive the issues of the *Journal of Pharmacy and Pharmacology*, as published, on preferential terms.

Members of any other Pharmaceutical Society, who are members of the Conference by virtue of the arrangement between their Society and the British Pharmaceutical Conference, shall not be required to pay a separate subscription to the British Pharmaceutical Conference; they shall be entitled, on application, to receive the *Journal of Pharmacy and Pharmacology*, as published, on preferential terms.

Other members elected by the Executive shall pay a subscription of 35*s*. annually, which shall entitle them, on application, to receive the *Journal of Pharmacy and Pharmacology*, as published. Subscriptions shall become due on January 1, and membership shall cease if subscriptions are not paid by June 1.

BRITISH PHARMACEUTICAL CONFERENCE

Newcastle upon Tyne 1960

CHAIRMAN: W. H. LINNELL*

SYMPOSIUM

CHEMICAL DISINFECTION

SOME ASPECTS OF THE DYNAMICS OF DISINFECTION

BY S. E. JACOBS, D.Sc., Ph.D., A.R.C.S.

Bacteriological Laboratories, Imperial College of Science and Technology, Prince Consort Road, London, S.W.7

THE course of the destruction of populations of bacteria by chemicals, and other lethal agents, can be followed by taking samples at intervals and determining the numbers of survivors by making colony counts on a suitable nutrient medium. With chemical disinfection it is necessary to ensure that the action of the chemical should be completely stopped when the sample is taken, either by the use of a neutralising agent or by dilution, for if this is not done there will be continuing action and the mortality obtained will be greater than that which existed at the moment of sampling. Here it is relevant to mention that nutrient media used for the cultivation of bacteria may contain substances which are lethal to cells already weakened, though not irreversibly damaged, by exposure to phenolic and other antiseptics, as shown by Jacobs and Harris[1] and Richards[2]. However, any errors which are thereby introduced are normally small and may be disregarded.

The idea that the survival curves of bacterial populations exposed to chemicals and other lethal agents could be expressed adequately in the form of straight lines by plotting the logarithm of the number, or the percentage, of survivors against time is of respectable antiquity, as it was first put forward in 1907 by Madsen and Nyman[3]. Instances where the experimental data deviated from the straight line were soon discovered; but these were usually explained away in terms of the presence of clumps of cells, or of populations of differing sensitivity in the supposedly homogeneous initial populations, and the ideal or true expression of disinfection data was held to be the straight line on this semilogarithmic graph.

In these circumstances it was not surprising that attention should be drawn to the similarity between the rate of a disinfection and that of a so-called unimolecular chemical reaction, where the "logarithmic law" also applies, and inevitably the analogy was pushed to its logical conclusion, which was that viable bacterial cells each contain a single molecule whose destruction or alteration results in the death of the cell. This idea was very strongly upheld by Rahn[4] as recently as 1943, but it was quite unsupported by direct evidence and it always appeared unlikely that so

* The Chairman's Address, entitled Education and Research, is published in the *Pharmaceutical Journal*, 1960, **185**, p. 227.

great a diversity of chemical agents could all act on the one molecule. Indeed, modern knowledge of the mode of action of disinfectants has made the theory wholly untenable and there is now no compulsion to believe that the "logarithmic law" must apply rigidly and in all circumstances. In fact, careful studies by various workers, including Withell[5] and Jordan and Jacobs[6,7], have revealed that a variation in type of reponse apparently occurs when the overall rate of the reaction is altered by changing the concentration of disinfectant or the temperature at which it acts, so that whereas in fast reactions the graph of log survivors against time is approximately a straight line, with slower and slower reactions there is an increasingly prominent initial phase of relatively low death rate. But Jordan and Jacobs[7] concluded that the variation was not a real one. Their analysis of a considerable body of data derived from an extensive study of the action of phenol on standard cultures of *Escherichia coli* under carefully controlled conditions suggested that the shape of the semilogarithmic disinfection curve was fundamentally the same in all circumstances. There was possibly a short initial period of fairly steep slope: but apart from this there was a period of low but steadily increasing slope, succeeded by a stage of maximum slope. Finally, there was a stage in which the slope again lessened. The transitions between these phases were gradual and the apparent change from an obvious curve to a straight line as the overall speed of the disinfection was raised was due to a gradual lessening of the differences between the slopes in the several phases. In addition, in fast reactions it becomes difficult to obtain experimental data relating to the first two of these phases because they are so quickly completed, and the mortality may have approached 90 per cent before the first sample can be taken. Clearly, in such circumstances the results cannot justifiably be used to fix the earlier parts of the disinfection curve.

At present comprehensive data have been published for one system only, namely *E. coli* and phenol, but it is known that the action of *o*-cresol on *E. coli* follows a similar pattern (Jordan, Jacobs and Penry, unpublished data). However, the establishment of disinfection curves calls for elaborate and time consuming experiments, and for that reason the technique is not suitable for the purpose of discovering how the behaviour of a disinfectant is affected by the conditions in the environment. Instead, it is customary to lighten the task by determining only "end points", that is, the times for apparently complete destruction of a population, which must therefore be of the same size in all experiments. Detailed discussion of the methods used, which are well known, would occupy too much space and seems unnecessary, and it is sufficient to point out that the end points correspond to the times required for the viable count to be reduced to some low but not in general accurately defined level of survivors. However, the mathematical treatment of end point data devised by Mather[8] yields a mean single survivor time, which is an accurately defined level of survivors.

Use of these methods has revealed that the rate of action of disinfectants may be greatly affected not only by concentration and temperature

but also by such conditions as the pH, the concentration and kind of the cations present, whether there is particulate matter in the environment and, most importantly, the kind and amount of organic matter present. It may be added that the way in which a bactericidal chemical is formulated may greatly alter its activity, but this aspect cannot be discussed here and it is sufficient to remark that the factors mentioned above will affect the rate of action of a chemical, whether formulated or not. It is also important to remember that the results will not be the same for any one chemical irrespective of the kind of organism used in the tests, and in view of all this it is evident that no useful assessment of the way in which a disinfectant will behave under the wide range of conditions likely to be encountered in practice can be obtained from the results of a single laboratory test. There is no alternative but to study the action of the disinfectant under a wide variety of conditions, and to make practical trials as well.

Useful as the end point methods are, it is nevertheless true that they yield only a fraction of the information about the course of a disinfection which is provided by the full disinfection curve, and in some circumstances a proper understanding of what has been happening in a culture exposed to a disinfectant cannot be reached unless the course of the reaction is followed in detail. Organic matter in the environment of bacteria is usually considered to have a protective action. It may react with the disinfectant and so reduce its concentration, or it may displace disinfectant from the cell surface, or may form a protective film. But it may have a further action and by serving as a source of nutrients and of energy assist the cell to maintain its structure and repair damage. Obviously this type of action could only be of significance if the other conditions were such that metabolism would be possible for some of the cells and for this to be so the concentration of the disinfectant would have to be small. This situation arose in experiments designed to determine the threshold concentration for phenol acting on standard cultures of *E. coli* at 35°. As will be seen from the results described below the value obtained was unexpectedly small, but because the full disinfection curves were determined support was afforded for a possible explanation.

The Predicted Value of the Threshold Concentration for Phenol Acting at 35° on Standard Cultures of E. coli

In the series of experiments carried out at 35° by Jordan and Jacobs[7] data were obtained showing the relation between percentage mortality and time over the phenol concentration range 3·48–8·00 g./l. From these the 99·999999 per cent mortality times (virtual sterilisation times) were calculated[9] and used to determine the concentration exponent for phenol, employing the linear relationship between log virtual sterilisation time and log phenol concentration.

The value obtained, using the full range of phenol concentrations, was 5·85 ± 0·19, and this agrees well with the value of 5·7 obtained by Tilley[10]. However, the virtual sterilisation time for 3·48 g./l. appeared rather too large, and when the concentration exponent was recalculated omitting

that result its value was reduced to 5·66 ± 0·14. Though this was not a significant reduction, the effect was to make the experimental value of the virtual sterilisation time for 3·48 g./l. even more divergent from the predicted value than it had been before. In fact it was now rather more than 30 per cent too high. This was not considered to be a reason for rejecting the lower value of the concentration exponent, but rather as an indication that 3·48 g./l. was close to the threshold concentration. At that point sterilisation takes an infinitely long time, so that the logarithm of the virtual sterilisation time must become infinitely large at a finite value of the logarithm of the phenol concentration. In other words, the graph of log virtual sterilisation time against log phenol concentration must steepen sharply as the threshold concentration is approached and the experimental values should be increasingly greater than those predicted by extrapolating the straight line which closely fits the data for higher concentrations. These relationships are illustrated in Figure 1, where line A is the straight line which best fits the data from 3·76 to 8·00 g./l. and line B is the hypothetical increasingly steep portion which should become asymptotic to a vertical line drawn through the logarithm of the threshold concentration.

The concept of the threshold concentration was subsequently developed further by Jordan and Jacobs[11], employing data obtained at different temperatures and a mathematical treatment which need not be described here, and it finally appeared likely that the value was close to 3·00 g./l. It was then decided to attempt to verify this prediction experimentally, using the same strain of *E. coli* as before, and a preliminary account will now be given of this work, which was carried out by Stone, Jacobs and Jordan.

Experimental Technique

This has been fully described by Jordan and Jacobs[1], but it is necessary to describe it briefly here because of its special features.

A standard culture of *E. coli* is developed in about 1,500 ml. of pH 7·0 phosphate buffer solution containing 1·0 g. of Difco dehydrated nutrient broth in a 5 litre round bottomed Pyrex flask held in a thermostatically-controlled water bath and agitated by means of a stream of sterile air. The dilute broth is inoculated with 300–400 viable *E. coli* cells per ml. and at the same time an automatically-operated syringe, which delivers small constant measured volumes of a solution of Difco dehydrated broth (6 g./l.) at 100 second intervals to the culture, is started. The rate of broth addition is 15·2 mg./hour. In these circumstances the bacterial population rises in a few hours to *c.* 330×10^6 viable cells per ml. and thereafter remains constant, being controlled by the rate of the continuing addition of nutrient broth. However, the total population, determined by culture turbidity, continues to rise, so that the culture consists of slowly dividing cells. After 40 hours from the inoculation the disinfection experiments are started by adding the required amount of 5 per cent phenol solution dropwise, the addition occupying 5 minutes. Thereafter samples are taken at intervals as required and the survivors counted by the

dilution plate colony count technique, with, usually, ten replicate plates per dilution.

For the attempt to determine the threshold concentration the technique was modified slightly. The initial population was *c.* 250 × 10^6 viable cells per ml. only, because it was realised that the experiments would in some instances be very long drawn out. This was achieved by reducing the initial broth addition from 1·0 g. to 0·5 g., though the regular addition

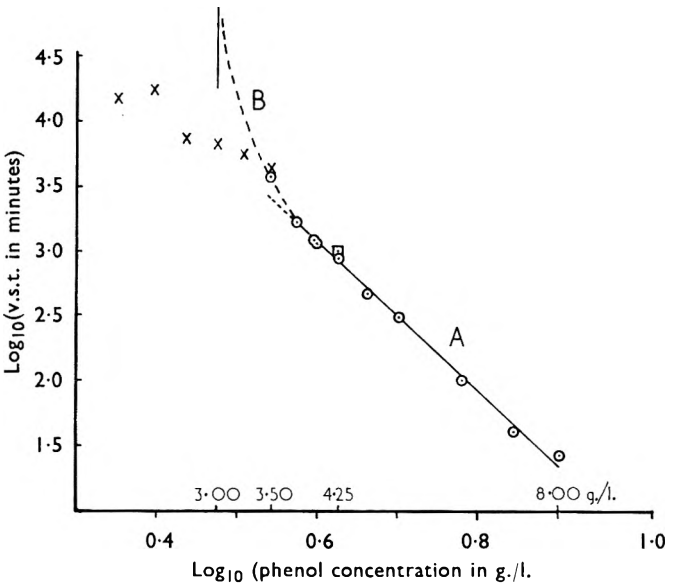

FIG. 1. The relationship between the logarithm of the virtual sterilisation time (v.s.t.) at 35° and the logarithm of the phenol concentration, for standard cultures of *E. coli*.

Circles, original data; square, newly obtained value, original conditions; crosses, newly obtained values, slightly modified conditions. The continuous line A is that which best fits the original data, its slope being 5·66 ± 0·14. The broken line B is the hypothetical continuation of line A and is asymptotic to an ordinate at the logarithm of the originally deduced threshold concentration, 3·00 g./l.

was at the same rate as before. Even so, some of the experiments lasted for over 10 days, and it was obviously necessary to ensure that the phenol concentration was not materially reduced in these long periods through losses in the air stream. Estimates of the actual loss were therefore made and the amounts made good by adding each day the required amount of 2 per cent phenol solution. The concentrations present in the cultures were checked from time to time by means of direct estimations and in no experiment was the actual concentration more than 3–4 per cent below the desired value at any time.

The concentrations used ranged from 2·0 to 3·50 g./l. in steps of 0·25 g./l. Whenever it appeared desirable an experiment was repeated, but the results were always substantially the same. In addition, the general level of resistance of the culture under the earlier experimental conditions was checked in an experiment at 4·25 g./l.

Results and Discussion

It is not possible here to present all the results in detail or to discuss all their features. This it is hoped to do in a later publication, but here only the salient features will be mentioned.

It is important to note that the organism used was still behaving substantially as it had in the earlier experiments, as judged by the virtual sterilisation times at 4·25 and 3·50 g./l. As shown in Figure 1, the logarithms of these were very close to the values expected from the earlier results, though actually the cultures were rather more resistant than before, the numerical values of the virtual sterilisation times both being about 16 per cent larger than expected. Despite this, however, the cultures were notably more sensitive than expected at concentrations below 3·50 g./l., and so far from the threshold concentration being close to

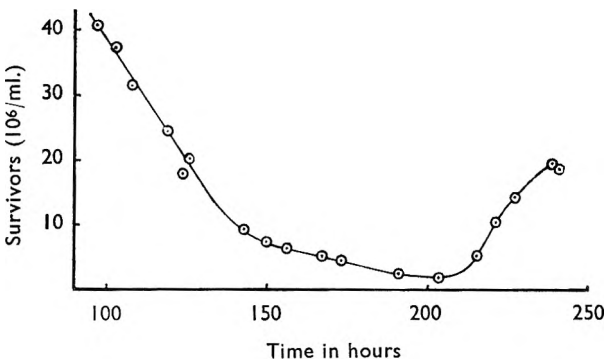

Fig. 2. The changes in viable cell count in the later stages of an experiment in which a standard culture of *E. coli* was exposed at 35° to phenol at 2·00 g./l., showing the ultimate occurrence of growth.

3·00 g./l. complete destruction was obtained down to 2·25 g./l. (Fig. 1). But not only was the threshold concentration lower than expected; the pattern of behaviour of the cultures had altered and instead of the expected steady rise in slope of the graph of log virtual sterilisation time against log concentration there was an increasingly wide divergence until suddenly, at 2·00 g./l., sterilisation was not complete. In fact, at that concentration, multiplication occurred after the viable count had fallen to about $1{\cdot}75 \times 10^6$/ ml., i.e., 0·83 per cent of its initial value (Fig. 2), and a further point of interest is that the virtual sterilisation time for 2·25 g./l. was not larger but smaller than that for 2·50 g./l. (Fig. 1).

It is evident that in these cultures some disturbing factor was at work, and consideration of certain features of the disinfection curves leads to the conclusion that the experimental conditions had permitted growth to occur, with accompanying alterations in the sensitivities of varying proportions of the cells in the cultures.

It is evident that some cells of a culture can grow in the presence of a concentration of phenol which is lethal to most cells, as shown by the

later part of the survivor curve at 2·00 g./l. (Fig. 2). The fact that growth occurred indicates that available food was present, and there is no doubt that the rate of food addition was for a long time in excess of the amount which could be utilised by the falling viable population. This was below 10×10^6/ml. for at least 60 hours before the growth commenced, and as in that time over 0·9 g. of food was added there should have been ample to support quite a large population. But the increase in viable count was not extensive and there are also signs that it had reached its limit at about 20×10^6 cells per ml. It is, therefore, perhaps remarkable that the growth was so restricted. However, a possible explanation is suggested by the

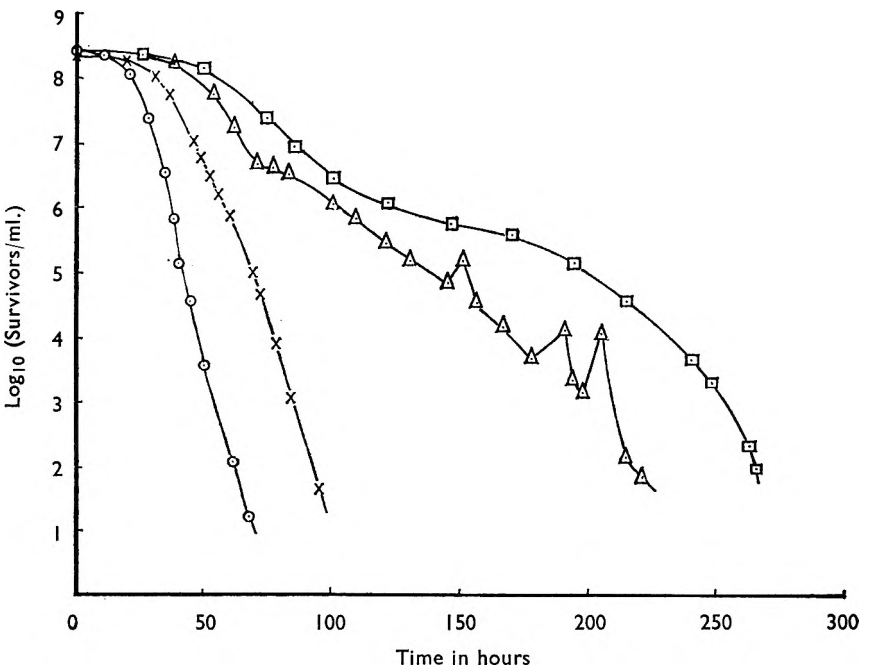

FIG. 3. The relationships between the logarithm of the number of survivors and time in standard cultures of *E. coli* exposed at 35° to phenol. The concentrations used, in g./l., were: 3·50 (circles), 3·00 (crosses), 2·50 (squares) and 2·25 (triangles).

fact that the increase in turbidity which occurred at this stage was much larger than the observed rise in viable cells could by itself have produced. The turbidity rose from 20 per cent below to 20 per cent above the initial value, and as the viable cell increase could have accounted for about one-sixth only of this, it seems clear that many more cells must have been formed, but had been killed. Thus rapid death must have accompanied the multiplication stage, and it is possible that had the experiment been continued the viable count might have dropped again and even ultimately reached zero. The possibility of this is indicated by the viable counts in the later stages of the experiment at 2·25 g./l., where marked sudden fluctuations occurred before the count finally reached zero, as shown in

Figure 3. This phenomenon has not been observed before in the now extensive series of experiments on these standard cultures. These particular fluctuations did not coincide with marked turbidity changes: but this is not surprising since they occurred at a time when the viable population was of the order of thousands per ml. only.

Since it appears that rapid death may accompany an increase in viable count in these slow disinfections, it seems logical to enquire whether multiplication may not also have been proceeding during the phase of continual decline in count, as it does in the decline phase of a normal culture[12]. Evidence is provided by abnormalities in the shapes of the graphs of log per cent survivors against time for 2·50 and 2·25 g./l. As shown in Figure 3, whereas the graphs for 3·50 and 3·00 g./l. were quite similar to the general pattern described above, at 2·50 and 2·25 g./l. there was a strongly marked intermediate period of high slope followed by a long period of low slope before a further steepening occurred. This change could have been due to the production of new cells, food and energy being available because of the lessened demand on the food being supplied and the phenol concentration not being too high to prevent the growth of all cells. It is also conceivable that even if cells were not multiplying they were succeeding in repairing the damage caused by the phenol and so increasing their survival time. However, the effect of cell repair and multiplication, it might be supposed, would be to decrease the overall rate of action of a given phenol concentration, whereas the virtual sterilisation times obtained were in fact much shorter than expected. Also, there are other indications of an abnormally increased sensitivity of the cells in these cultures in certain circumstances, namely the marked relatively rapid cell death in the intermediate stages at 2·50 and 2·25 g./l. There is also the fact that 2·25 g./l. was not less but more effective than 2·50 g./l. (Fig. 3).

Now while no fully comprehensive explanation of the experimental results can be offered, much of the behaviour of these cultures, in which the conditions were not only complex but changing in different ways in different circumstances, can be accounted for with the aid of two concepts: first, that the inherent ability of a cell to withstand damage varies according to the stage it has reached in the division cycle, which implies that at a particular stage resistance is minimal; and second, that after fission in the presence of phenol the daughter cells are more susceptible than the parent. The first concept, which has already been put forward by Hinshelwood[13] to explain the abnormal protraction of the final stages of decline in supposedly uniform populations exposed to lethal agents, is difficult to reconcile with the occurrence of an almost straight line relationship between log survivors and time, as pointed out by Eddy[14]; but this difficulty does not exist where that relationship does not hold, as in the present work. It must be added that for the present purposes the level of resistance at the minimum is considered to differ among the cells of a culture, and indeed it may be supposed that some cells may always display a higher level of resistance than others at corresponding stages of the division cycle.

By level of resistance is meant the period of exposure the cell can tolerate in the absence of an external supply of nutrients and still be viable when transferred to a nutrient medium. If nutrients are available during exposure repair may be possible and survival prolonged, and if the supply is sufficiently large and the phenol concentration sufficiently low growth may occur and the cell will proceed through the division cycle. But according to the initial position of the cell in that cycle it may become either more or less resistant. If the latter, death may occur sooner than it might otherwise have done, while cells whose resistance was increased may succeed in dividing, only to be brought in their turn towards the condition of minimal resistance and to their death. In this way an accumulation of food may not only accelerate death but induce multiplication followed by accelerated death. However, if daughter cells are always closely similar to their parents in resistance level some cells of high resistance would be expected to survive in low phenol concentrations and continue to multiply till a new equilibrium with the food supply was established. But that did not occur in the present experiments and there were apparently spurts of multiplication followed or accompanied by rapid death. To account for these findings a progressive increase in inherent sensitivity following division of cells in the presence of phenol has been postulated. In this connection it may be recalled that Van Eseltine and Rahn[15] observed transient waves of growth in broth cultures of *E. coli* containing low concentrations of phenol.

This consideration of the results of experiments conducted on cultures in which the conditions were changing in a complex way has necessarily been superficial, but the ideas introduced do make it possible to imagine how a lower concentration could be more effective than a higher one, either at all stages of a disinfection process or at certain stages only, in terms of an interaction between the effects of phenol and of nutrients on the metabolism of individual cells at different stages of the division cycle. The further idea of a general increase in sensitivity of cells following division in the presence of phenol is not an unreasonable one, for such cells might easily be less well endowed with reserves than normal cells.

In conclusion, it may be remarked that whereas organic matter is known to protect bacteria against disinfectants the experiments described above have shown that in certain circumstances, for example, at disinfectant concentrations approaching the threshold value, utilisable organic matter may make a culture as a whole more sensitive. The experiments failed to fix the threshold concentration for phenol acting on *E. coli* at 35° because the conditions changed too greatly during the disinfections. It will probably be impossible to adjust the food supply exactly to the changing demands as cells are killed and so to make possible an accurate determination of the true threshold concentration.

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PHENOLIC DISINFECTANTS

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Phenol was discovered in 1834 but it was 1860 before it was first recommended as a disinfectant[1] and, in the same year Lemaire[2] used it on wounds. In 1867 Lister[3] introduced it in antiseptic surgery and in 1877 Jeyes patented what must have been the first coal tar disinfectant, which was a creosote-soap solution.

Because of Lister's work and as phenol itself was comparatively easy to obtain in a pure form, it was adopted as a reference substance in disinfectant work about the beginning of the century and is still accepted as such in most of the disinfectant industry.

Its toxicity, especially its leucocidic power which is involved in sloughing of wounds, prevented its wide use as an antiseptic.

Mode of Action

It has been stated that phenols act by denaturing bacterial proteins, and this idea of direct chemical action by coagulation of proteins is supported by Bancroft and Richter[4] and Lobes[5].

As early as 1909 Reichal[6], after studying the distribution coefficients of phenol between oil and water and their relation to bactericidal activity, suggested the action to be physical rather than chemical, an idea that found support from Richardson and Reid[7].

Pulvertaft and Lumb[8] noted that lysis of bacteria often occurred in presence of bacteriostats, and Gale and Taylor[9] studied the leakage of glutamic acid from phenol-treated bacteria and concluded that phenol alters the permeability of the cell wall and allows essential cell material to leak out. Whether this leakage causes death or death precedes the leakage is not known, but the work of Bean and Walters[10] on the growth of phenol-treated organisms in the presence of the eluate from phenol-killed organisms suggests that death may precede lysis.

The work of Quastel and Wooldridge[11], Bach and Lambert[12], Sykes[13] and other workers, has shown that some bacterial enzymes are not completely inactivated by phenol at concentrations above those lethal to the organism itself, indicating that it is unlikely that the bactericidal action follows from complete enzyme inactivation.

Evaluation of Phenolic Disinfectants

Many adverse criticisms of the use of a phenol coefficient as a measure of bactericidal activity have been raised[14–17] but these have had little effect within the field of commercial disinfectants. Here, far too much importance is still attached both by manufacturers and customers to the Rideal Walker, Chick Martin, and Food and Drug Administration Phenol Coefficients. The tests used to ascertain these 'coefficients' are all of the extinction type and use phenol as a reference substance. The variable factors are numerous, some are controlled, often inadequately, by the specifications for the tests. Very little or no replication is suggested in the specifications. In the design of the tests, and in the calculation of

the phenol coefficients, no regard is paid to the assessment, of extinction time data, of the type suggested by Mather[18], and the use of such tests for other than batch to batch control is fundamentally unsound.

Unfortunately the published results of the "evaluation" of phenolic disinfectants abound with "phenol coefficients" the conditions of determination of which, in many instances, are not stated. Thus it is difficult to draw conclusions from, or make comparisons between, the results from different laboratories when evidence about the conditions attaining; or where the repeatability and reproducibility is not available. An example of this difficulty is given by Coulthard[19] who in assessing the phenol coefficients of 4-n-amyl-*m*-cresols suggested that the differences between his results and those of other workers could be attributed to the use of alcohol or alkali to maintain the phenols in solution.

Although it would be fallacious to compare the bactericidal value of different phenols by comparing phenol coefficient values, especially where these were from different laboratories, it is possible to note gross differences and trends.

Factors affecting Action of Phenolic Disinfectants

Concentration. Tilley[20] obtained values of between 7 and 9 for the dilution coefficient of several phenols against *Salmonella typhi* and *Staphylococcus aureus*, the higher molecular weight homologues giving slightly higher values than phenol. These values are higher than many other classes of disinfectant and make the Use Dilution Confirmatory Test seem pointless since with a phenolic disinfectant with a phenol coefficient (F.D.A. Test) of 3 then the concentration of the disinfectant used in the confirmatory test should kill the organism in less than 1×10^{-4} minutes. In the test 10 minutes is the permitted reaction time, a safety margin of 100,000. On the other hand one dessertspoonful of solution of chloroxylenol in a handbasin of water would need 3 days' contact time to approach disinfection of the objects immersed, if the dilution effects depended solely on the chloroxylenol content.

When phenolic disinfectants are used in practice these high dilution coefficients demand that care be taken not to dilute beyond an effective concentration.

Temperature. There are two aspects of the effects of temperature on disinfection by phenols. The first is that the bactericidal activity of phenols increases rapidly with increase in temperature. The second follows from the work of Grubb and Edwards[21] that some strains of *Salm. typhi* and *Staph. aureus* are more resistant to phenol when the cells were grown at higher temperatures, which means that a higher concentration of a phenolic disinfectant may be necessary to disinfect material contaminated by bacteria from human sources than if contaminated with soil organisms.

Hydrogen ion concentration. In general phenols have greater antibacterial activity at an acid than at an alkaline pH[22,23]. With an amyltricresol the antibacterial activity against *Staph. aureus* decreased with increased pH whereas with *Escherichia coli* it increased with pH[23].

Oxidation-reduction potential. Gould, Frigeris and Hovanesia[24] and other workers have shown that anaerobic organisms are usually more resistant to phenols than aerobic organisms. Their findings with facultative organisms (bacteria that are able to grow with or without oxygen) are perhaps more significant, in that these organisms are more resistant to phenols under anaerobic conditions.

The ability of some bacteria when grown in conditions of low redox potential to oxidise phenols was first reported by Wagner[25] and more recent work [26] concludes that there are diverse groups of bacteria which can oxidise phenols under a variety of conditions.

Development of resistance to phenols. There are conflicting reports on the abilities of bacteria to acquire a resistance to phenols[24,27,28] but the reported resistance was not very great and was not retained for long periods if subcultured in the absence of the phenol.

Organic matter. It is well known that organic material can interfere with the antibacterial activities of phenols, a fact recognised in the design of the Chick Martin Test. This interference is stronger in some other kinds of disinfectant, for example the quaternary ammonium compounds. It also varies with different phenols and different kinds of organic matter.

Chemical nature of phenols. Dihydric and polyhydric phenols are generally less active than monohydric phenols.

Coulthard, Marshall and Pyman[29] have shown that alkylation of monohydric phenols potentiates their activity. This effect is maximum where there are 5 carbon atoms in the substituent group.

A similar enhancing effect is produced by halogenation of phenols and increases with increasing atomic weight of the halogen; it is less in the *ortho* than the *para* position[30]. This increase in activity on halogenation generally has the effect of an increased specificity of action against the different genera of bacteria.

Increase in molecular weight of the phenol is usually accompanied by decrease in solubility, and this has led to the use of soaps in the formulation of phenolic disinfectants to bring insoluble phenols into solution.

Cresol B.P. is a mixture of *ortho*, *meta* and *para* cresols together with small amounts of xylenols. It is more bactericidal than phenol, less toxic, and less soluble. Pentachlorphenol is extensively used in timber preservation and has an odour too strong to recommend its use in disinfectants. Chlorocresol B.P. used as a bactericide and bacteriostatic in pharmaceutical preparations has slight irritant properties. Chloroxylenol B.P. is only slightly soluble in water and is used extensively in preparations similar to Solution of Chloroxylenol B.P. This preparation was designed for use against haemolytic streptococci, it has very poor action against staphylococci and pseudomonads; it is to be regretted that it has been recommended as a substitute for lysol for general disinfection as it is often used in concentrations which are quite inadequate. Dichloroxylenol has been suggested as a substitute for or adjunct with chloroxylenol. It is more bactericidal than chloroxylenol when tested against salmonellae, staphylococci and streptococci but is ineffective against pseudomonads.

Phenols and Soaps

Since the patent by Jeyes in 1877 soaps have been an almost constant feature in phenolic disinfectant formulae. The earliest pharmacopoeial formula was that of lysol in the 1914 B.P. Lysol is still widely used but the production of a standard poses a problem. The official monograph is so loosely drawn that variations in bacteridical power can occur which are too large for this important preparation. The soap content plays an important part. Berry and Stenlake[31] have shown that the bactericidal value of a lysol depends upon the nature and amount of the soap used. With the same sample of cresol, variations occurred ranging from 1·4 to 3·2 when measured as the phenol coefficient against *Salm. typhi.* Moreover the raising or lowering of the coefficient using this organism is not reflected in a similar effect using other organisms such as streptococci or staphylococci. It can also be shown that an optimum effect can be obtained in the presence of organic matter by a careful selection of the soap or blend of soaps. The official monograph however permits the use of any sodium or potassium soap or mixture to be used providing the physical characteristics of the preparation are maintained. The formulation for Liquor Cresolis Saponatus B.P. 1914 would produce much less variation, but economically it could not compete in price with the present formula recommended by the trade.

Berry[32] is correct in stating that by raising the standard of the chemical and physical specifications of the cresol and the soap used in making lysols more reproducible bactericidal values for the lysols would follow. It is recognised that a good standard lysol could be prepared from a pure *ortho*, *meta* or *para* cresol and a specified soap but such a lysol would have a poor reception in the economic field because of cost. Nevertheless there is room for a further effort either by the Pharmacopoeial authorities or the trade itself to improve the standardisation of this important disinfectant, the use of which is on the increase. Lysol has a wide bactericidal spectrum including activity against *Pseudomonas pyocyanea.* It undoubtedly plays an important and valuable role in hospital practice and general hygiene and because of this should receive attention. Its greatest limitation is its irritant effect on the skin, but "lysols" have been formulated which are much less irritant than those using cresol.

Many attempts have been made to explain the effect of soap on the bactericidal activity of phenols, and results are still contradictory.

Soaps are surface-active agents which exhibit the phenomenon of micelle formation. McBain[33] postulated that in dilute aqueous solution soap behaves as a normal electrolyte, but at higher concentration reassociation takes place to form "micelles." These he regarded as being spherical and consisting of an aggregate of the hydrophobic hydrocarbon chains of the soap molecules jumbled together and away from the water with their hydrophilic end groups projecting into the surrounding water and the whole aggregate surrounded by an atmosphere of the hydrophilic ions of the soap. Stauff[31] postulated lamellar micelles consisting of double layers of soap molecules closely packed side by side. These micelles begin to form when the concentration of the soap reaches the critical

micellar concentration. With a sample of pure potassium laurate, this occurs at about 0·03 M and the micelles increase in size and number until 0·05 M. The critical micellar concentration is a characteristic of each soap under constant conditions but is affected by temperature, presence of electrolytes and other substances, for example, hydrocarbons.

The most important property of these soap solutions, in relation to phenolic disinfectants, is their ability to solubilise insoluble phenols in the micelles, and the effect this has on the bactericidal activity of the resultant solution.

Figure 1 shows typical results obtained by workers in this field[35]. This shows (dotted line) the solubility of a comparatively water insoluble

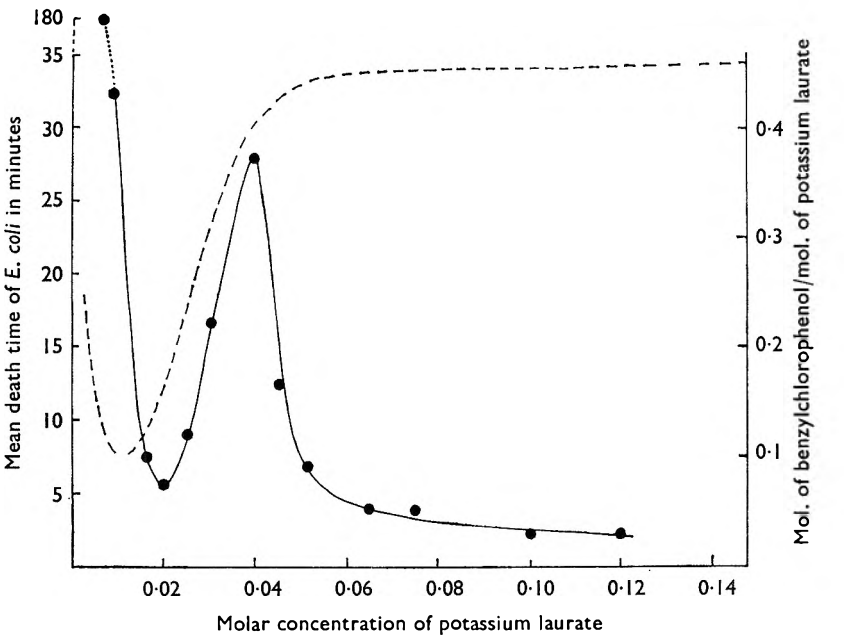

FIG. 1. Solubility of benzylchorophenol in potassium laurate and bactericidal activity of solutions containing a constant molar ratio of phenol to soap of 0·0653 (after Berry and Briggs). Broken line is solubility curve.

phenol in solutions of potassium laurate and the death time of *E. coli* in a solution with a constant phenol to soap ratio with differing soap concentrations. It shows that the death time decreases with increasing soap concentration up to and just above the critical micelle concentration. Then there is a rapidly increasing death time up to about 0·04 M which thereafter decreases.

Alexander and Tomlinson[36] used Aerosol MA, an anionic surface active agent, to solubilise a constant concentration of phenol with varying concentrations of the solubiliser. Their results are not very explicit. Thus when their concentration was above the critical micellar concentration they did not get an inflection in the curve indicating minimal activity

as reported by Berry at 0·04 M potassium laurate, and they postulated that the curve continued upward until it met the curve representing the death times in aerosol MA alone.

In an effort to elucidate this apparent contradiction, Berry, Cook and Wills[37], used potassium laurate and three phenols of differing solubilities, and plotted similar curves both at constant phenol to soap ratios and constant phenol concentrations (Fig. 2 and 3). They confirmed the presence of the peak and showed that there was also a smaller second peak with the constant phenol concentration mixtures, this second peak was highest with the most water soluble phenol. With the constant phenol to soap ratios they confirmed the presence of the first peak but it was least well marked with the most water soluble phenol.

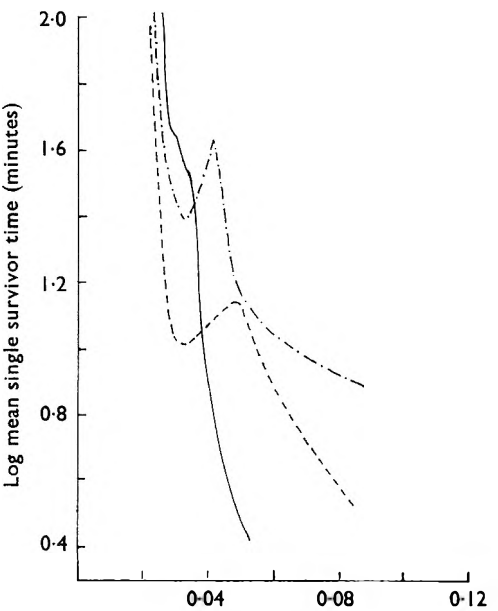

FIG. 2. Bactericidal activity of solutions of phenols in potassium laurate solutions with a constant phenol/soap ratio.
—— Phenol; – – – 2-hydroxydiphenol; –·–·– 4-benzylphenol.

Alexander ascribed the effect of the solubiliser to the formation of an interfacial complex at the bacterium-water interface, and, at concentrations in excess of the critical, the phenol passed into the micelles and the activity was that of the solubiliser itself.

Berry's explanation of the peak was that soap micelles and bacteria were competing for the phenol. When the soap micelles were saturated with phenol the increased activity was re-established.

These two explanations adequately cover the findings reported in the respective papers and are in some ways complementary. Both agree about the rapid decrease in death-time with increasing concentration until

the critical micellar concentration. Above this concentration, there is a difference between systems with a constant phenol concentration (Alexander) and a constant molar ratio of phenol to soap (Berry). The later work[37], with a constant molar ratio, supports Berry's earlier explanation but results with a constant phenol concentration, where the micelles are not saturated with phenol and the inflections in the curve still appear, detract from this explanation. More information about the distribution of phenol between micelles and the surrounding medium should help to answer this problem.

From the point of view of practical disinfection the results at constant phenol-soap ratio are more important, since this represents the dilution of

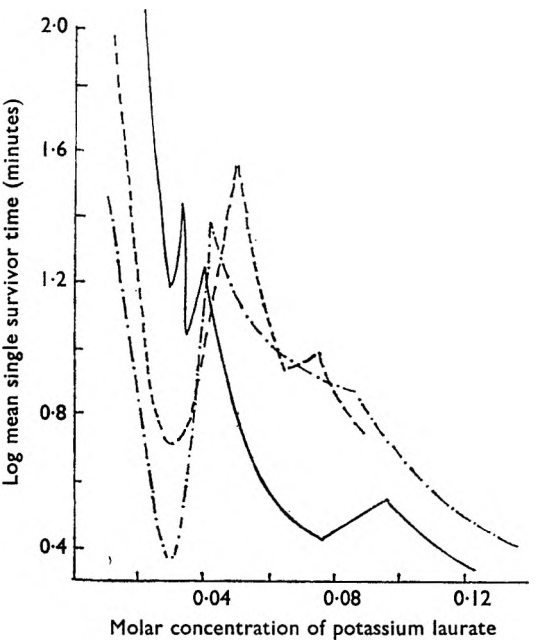

FIG. 3. Bactericidal activity of solutions of phenols in potassium laurate solutions with constant phenol concentrations.
—— Phenol; – – – 2-hydroxydiphenol; –·–·– 4-benzylphenol.

a concentrated disinfectant with water. Unfortunately, the findings are difficult to apply to commercial disinfectants for a number of reasons. These are summarised.

1. The critical micellar concentrations of different soaps are different, and this affects the bactericidal activity[3].

2. In many commercial disinfectants the soap used is impure or is formed from a hydroxyacid. In these circumstances, estolides may be formed[38] and so the critical micellar concentrations may vary from batch to batch.

3. The critical micellar concentration varies with temperature and alters when other substances, such as electrolytes, hydrocarbons and

alcohols, are added and most formulations contain one or more of these substances.

4. With many commercial disinfectants, especially the coal-tar group, the composition of the phenols varies from batch to batch.

Where the concentration of the disinfectant that is to be used is greater than that of the critical micellar concentration, then no great advantage is to be gained by any further knowledge except that it may be possible to dilute the substance further and get similar or greater activity.

If the Rideal-Walker test is used as an assessment of the disinfectant, then anomalous results are inevitable if dilutions around the critical micellar concentration have a death time of about 7 minutes.

A great deal of information is needed on the critical micellar concentrations of different soaps and the effects of added substances before true assessment of these disinfectants can be attempted.

Phenolic Disinfectants

Most commercially-available phenolic disinfectants are the coal-tar disinfectants. The aim of the manufacturer here is to offer a concentrated solution which can be diluted for use. Since, as mentioned earlier, many of these phenolic substances are insoluble, concentrated solutions must be formulated. This leads in turn to the main classes, the first of which is (*a*) the clear fluids which, on dilution, give clear solutions or emulsions, and (*b*) the concentrated emulsions which are stable on dilution.

The phenols which are used are classed as coal-tar derivatives.

There are three main types of carbonisation of coal—in all of which the tar is of secondary importance.

1. Low temperature carbonisation (for smokeless fuels), the phenols are separated from the tar and used, but the hydrocarbons which are present have too high a paraffin content to be of much use in disinfectants.

2. Vertical retorts in which the hydrocarbons contain about 20 per cent of paraffins and hence are of limited use.

3. Horizontal retorts (coke ovens) in which the hydrocarbons are almost free from paraffin.

Tars from the various methods are often mixed for distillation purposes. Low temperature and vertical retort phenols usually have a higher proportion of polyhydric phenols present which become discoloured, especially in alkaline solution.

Modern fractionation methods usually collect the phenol, *ortho*-cresol, a mixture of *meta*- and *para*-cresols, the xylenols and ethyl phenols in a high degree of purity. The main use of these is in the plastic industry with a smaller use for cresol used for lysol.

The middle oils, distilling between 205°–230°, are usually washed with alkali to leach out cresols and phenol which are separately recovered, and the rest is used for the preparation of low coefficient black fluids which are often reinforced with xylenols.

The high boiling tar acids (HBTA) distilling over 230° are very complex, usually containing more than 30 different phenols. The HBTA from low temperature carbonisation tars which contain a high proportion of

polyhydric phenols are usually treated to remove these. The HBTA from vertical retort tars are used without treatment, but they often redden if no antoxidant is present in the formula and so give what is sometimes known as red-emulsion type black fluids. The HBTA from horizontal retort tars give white-emulsion type black fluids. The hydrocarbons from the horizontal retort tars are used as carriers in black fluids.

Black fluids contain about 20 per cent of water and are clear solutions of coal-tar phenols with hydrocarbon carriers solubilised by a suitable soap. Resin, castor oil, palm kernel, coconut, fish oil or naphthenic acid soaps are used. White fluids are concentrated emulsions of the phenols stabilised with protective colloids such as glue and contain 45 per cent or more of water.

Black fluids dilute to emulsions with soft waters whereas white fluids dilute with hard or soft waters.

Black fluids are more stable on storage. A black fluid made with the same phenolic fraction as a white fluid is more bactericidal. The bactericidal activity increases with increase in phenol content, with the boiling range of the phenol used, and can also be increased by careful addition of carriers, which are usually aromatic hydrocarbons, especially in white fluids and soap-based fluids.

Organic matter reduces the bactericidal activity (based on Chick Martin test) of all fluids but those based on HBTA and those with added carriers are most affected.

The bactericidal power of lysol, which may be regarded as the simplest black fluid, varies with the soap used to formulate it, a useful point to remember is that soaps that give lower bactericidal activity usually give less opalescent solutions when the lysol is diluted.

Formulations of the Solution of Chloroxylenol type can be regarded as special cases of black fluids but, as stated earlier, the use of chlorinated phenols, whilst increasing the bactericidal power against some organisms, usually produces in a narrower specturm.

Hydrocarbon oils are used as "carriers" in black and white disinfectant fluids. Alone, they have no bactericidal value but they enhance the effect of the phenols. The phenolic fraction dissolves in the carrier which forms the disperse phase in the white fluids and is solubilised by the soap in the black fluids from which it is thrown out of solution on dilution.

It is to be regretted that the bactericidal activities of these formulations have been judged by phenol coefficient tests against *Salm. typhi.* HBTA, as was stated earlier, do show specificity and *Salm. typhi* exhibits higher specific sensitivity to HBTA than to phenolic fractions of lower boiling point.

There is little published information on the sporicidal activity of the phenols, and it has been suggested that some bacterial spores will survive for long periods in 5 per cent phenol, but all attempts by the author to isolate such a spore have so far proved unsuccessful.

Uses of Phenolic Disinfectants

Phenolic, especially the coal-tar disinfectants, are amongst the cheaper preparations, and having a broad spectrum they can be recommended as

good general disinfectants. But, because of their irritant properties, prolonged contact with the skin or mucous membranes should be avoided.

In most problems of cross-infection, staphylococci are implicated and, because some results show chlorinated phenols and HBTA to be comparatively less effective against staphylococci than formulae based on unchlorinated phenols of lower boiling point, the latter should be used.

Phenols, too, are less affected by organic matter than other types of disinfectant, and the soaps used in their formulation have detergent properties.

In conclusion, since, it seems likely that phenolic disinfectants will retain their place as good general purpose disinfectants, continued research to elucidate the problem of soap-phenol solutions, mode of action, and methods of evaluation are well justified.

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CHEMICAL STERILIZATION

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Physical conditions such as heat and radiation, and chemical substances which destroy micro-organisms are the two broad groups of agencies used for sterilisation or disinfection. The main fields of application are medicine and public health, the food industries, especially those concerned with highly perishable foods such as milk, and also water.

The classical meanings of the words "disinfection" and "sterility" are still valid, but new terms have been introduced and the classical terms are sometimes used with a modified meaning. Thus the terms "antiseptic", "disinfectant" and "sterilant" are used for similar substances, and "disinfection", "sterilisation", "commercial sterilisation" and "near-sterilisation" for similar processes. Whereas the term "disinfectant" usually implies a powerful germicide with no specific qualifications, "antiseptic" usually implies a germicide with negligible irritant effect on the tissues. The term "sterilant" is used in the food industry, possibly because "disinfectant" so often implies a strong smelling phenolic substance.

Sterility means "incapable of proliferation", and so in the hygienic sense the complete absence of all forms of life. The word is sometimes used more loosely to indicate absence of pathogens, or absence of food spoilage organisms, the small numbers of harmless organisms being ignored. The word sterility should *always* be used in its strict sense; sterility, like virginity, cannot be qualified. A thing is either sterile or not.

In practice the destruction of pathogens or spoilage organisms is often sufficient, and the terms "disinfection", "commercial sterilisation" and "near-sterilisation" are then used. The first is accepted in medical work, and of the other two the latter is to be preferred. Appropriate tests are examination for surviving pathogens, where *E. coli* type 1 is sometimes used as an index which implies dangerous contamination, and for surviving spoilage organisms a maximum total count may be used as a convenient index.

Cleaning and Sterilising—Cleansing

The modern fashion of chemical disinfection has tended to reduce the significance of cleaning, which is of the greatest importance in all aspects of sterilisation. Cleaning may be defined as the complete removal of all extraneous material and especially organic matter, which is food for bacteria. Heat may in time penetrate a film of milk-stone, dried-on-egg, serum or pus, and kill any micro-organisms present, but such films may protect organisms indefinitely against all types of chemical sterilants or radiation. Apart from this, efficient cleaning removes mechanically about 99 per cent of the bacteria on a dirty object, and is, therefore, an integral part of the sterilising process. Cleaning and sterilisation, whether by heat or chemicals, are complementary processes. The term "sanitisation" is commonly used in North America for this combined treatment,

but this term, although etymologically correct, is not popular in Britain. A convenient term for the combined treatment is "cleansing".

In the food industries cleansing may be defined as "the complete removal of all extraneous matter and reduction of micro-organisms to 1 colony per sq. cm. area or per ml. capacity by standard tests"[1]. Organisms surviving are almost invariably spores and resistant cocci of no pathogenic or industrial significance. The validity of this practical definition is well borne out by the fact that when swab and rinse test results are less than 1 per sq. cm. or ml., presumptive coliforms are very rarely, if ever, found. Absence of this most useful index organism usually indicates that all ordinary pathogens and most spoilage organisms have been killed. Spore forming organisms are an unfortunate exception (cf. p. 31 *T*) and when destruction of these is necessary, much more drastic methods must be

TABLE I

SUMMARY OF STANDARD METHODS FOR CLEANING AND STERILISING

	Plant	Tanks and tankers	Pipelines (mains)	Cans	Bottles
Pre-rinsing ..	Cold water	Cold water	Cold water	Cold or warm water	Cold or warm water
Cleaning ..	1. Scrubbing with detergent. 2. Recirculation with detergent 3. Recirculation with ½–1 per cent nitric acid	Scrubbing with detergent	As for plant	Jetting with or without detergent (brushing)	Jetting or soaking with detergent
Rinsing after detergent ..	Hot water	Hot water	Hot water	Hot water	Warm water
Sterilising ..	Steam, hot water, chlorine, 'quats', nitric acid	Chlorine, 'quats', hot water, steam	As for tanks	Steam ('quats', etc.)	(Hot detergent sterilises) (steam)
Final rinsing ..	Water—if chemicals used	Water—if chemicals used	Water—if chemicals used	—	Water (unless steamed). May be chlorinated 5 p.p.m.

employed. Standard methods for cleaning and sterilising food equipment are given in Table I and precautions to be observed with various materials in Table II.

Bactericides and Bacteriostats

The generally accepted definitions are that a bactericide kills bacteria, whereas a bacteriostat merely prevents growth or proliferation. In practice this distinction may be difficult to sustain: vegetative cells of bacteria which cannot grow usually die, although sometimes very slowly. The difference in effect may be a matter of time, concentration and temperature. Proliferation of cells as measured by colony counts may not be the only form of growth of bacterial cells. All methods of assessing bactericidal and bacteriostatic power are dependent on the technique used. For practical purposes we may assume that if cells cannot be recovered by growth tests after removal of the chemical, then the action is bactericidal. If cells are recovered and grown, then the action is bacteriostatic. It is often difficult to decide when a cell is dead, and this is only one of many

reasons why laboratory tests may be an unreliable guide to the efficiency of a germicide in practice.

Chemical Sterilisation Compared with Steam and Boiling Water

Where feasible steam or boiling water, or both, are usually the best agents for sterilisation in industry. However, circumstances are often such that chemical sterilisation is preferred. Thus the use of heat may be impossible, as for human skin and tissues, for equipment made of thermolabile material such as glass or plastics, or for heat-sensitive components or finishes. Heat is difficult to apply to large surfaces such as open tanks and walls. It may not be economic if items at various points have to be sterilised rapidly at various times of the day and night. Chemical solutions can easily be stored in concentrated form in any convenient place, and are always ready for use at a moment's notice. An adequate

TABLE II

SPECIAL PRECAUTIONS

	Cleaning	Sterilising
Steel	Any alkali	Not chlorine
Stainless steel	Nitric acid can be used	Chlorine if pH 9 or above. Nitric acid possible
Aluminium	No acids or caustic alkalis. At least 25 per cent silicate	Not chlorine
Glass and coatings	Anything except strong alkalies	Anything. Sudden and considerable temperature changes undesirable

supply of steam may not always be available. If equipment is required to be used cold immediately after sterilising, steam and boiling water are obviously unsuitable.

Quite apart from convenience and efficiency, chemical sterilisation is often cheaper than steam. As with detergents, sterilising solutions can be run from equipment requiring the most thorough cleansing to other equipment where the standard required is not so high.

Sterilants Compared with Heat for the Destruction of Spores

Spores constitute a special problem in sterilising work, and unfortunately some spore-formers are extremely important, for example, *Cl. tetani* and *B. anthracis* in medicine, *Cl. botulinum* in canning, and *B. cereus* in pasteurised milk. The only effective ways of destroying spores with certainty are by autoclaving, for example at 120° for 20 minutes; or by dry heat, for example 170° for 2 hours; or by high concentrations of suitable germicides at higher temperatures than those that are adequate for the cleansing of equipment.

The ordinary treatments which are satisfactory for vegetative cells may be useless against spores.

COMMONLY USED STERILANTS IN INDUSTRY

Hypochlorites. Of all sterilants, chlorine or hypochlorite is the best from most points of view. It is cheap, convenient to use, powerful and has a wide antibacterial spectrum. Its greatest disadvantages are

corrosiveness and odour. The former can be minimised by using in cold solutions and at pH 9 or higher, but it is more bactericidal at neutral or acid reactions. Commercial preparations usually contain 9 to 12 per cent and domestic preparations about 1 per cent of available chlorine. Complete immersion in 150 to 200 p.p.m. hypochlorite will sanitise clean metal and glass in 2 minutes; 10 minutes gives an ample margin for sterility.

Quaternary ammonium compounds. Next to hypochlorite the quaternary ammonium compounds are probably the most useful. They are convenient to use, of very low toxicity to animals, without appreciable odour

TABLE III

STERILISING AGENTS—ADVANTAGES AND DISADVANTAGES

	Steam	Hot water (190° F.)	Chlorine	Quaternary ammonium compounds
Cost	Varies	Varies	Low	High
Convenience	Depends on layout	Recirculatory system necessary	Very convenient	Very convenient
Penetration	Good if adequate supply	Good	Clean plant essential	Clean plant essential
Heating effect	Tanks, etc., may require hours to cool Undesirable stresses may be set up	Less than steam	None or slight	None or slight
Suitability	Very suitable for enclosed systems and small articles in chests	Very suitable for pipelines (mains)	All purposes	All purposes
Persistence	Not persistent	Not persistent	May persist if traces chlorine, etc., remain	
Corrosion	None	None	Extremely corrosive especially at acid and neutral reactions	Not corrosive if thoroughly rinsed away
Odour	None	None	Very marked	None
Toxicity	None	None	Precautions essential	Not toxic in practice

or taste, non-corrosive, and not too expensive. They are very powerful against Gram-positive but are nearly always less effective against Gram-negative organisms, and this is their greatest disadvantage. They are incompatible with soaps and anionic detergents, but can be used with most alkalies and non-ionic detergents. Alkalinity increases their bactericidal power.

They are only very weak detergents. The relative advantages and disadvantages of steam, hot water, chlorine and the quaternary ammonium compounds are given in Table III.

Working concentrations vary from 1 in 1,000 to 1 in 20,000 according to the particular quaternary compound used, the temperature, the time, the alkalinity and the percentage kill desired. Weight for weight they are less effective than chlorine, but their other advantages may outweigh this fact. For detailed information on the quaternary compounds see Glassman[2], Lawrence[3] and Resuggan[4].

Alkalis. Although generally regarded only as detergents, caustic alkalis have, in addition, a strong germicidal activity. Thus in bottle-washing, bottles emerging from the detergent section, 3–5 min. in 0·4 per cent NaOH (equivalent) at 145° F., are usually sterile or nearly so[5].

The bactericidal effect falls rapidly with decreasing pH and is influenced by the nature of the anion (Table IV).

Hot caustic soda solutions at 1–3 per cent are specially useful for killing spores, and this is an advantage claimed for the soaker type of bottle-washing machines.

Iodine. Although very useful as an antiseptic, iodine has certain disadvantages as a sterilant. It has a powerful odour, stains badly, is not very soluble in water, and is expensive. It is readily soluble in iodide solutions and in alcohol, but this method increases its cost, and the solution may be irritant to skin.

Iodophors. Although of recent introduction, it is probable that iodophors will grow in popularity. They are prepared by the action of iodine

TABLE IV

BACTERICIDAL ACTIVITY OF ALKALIS

	pH of 1 per cent solution	Killing time in minutes against *B. subtilis* spores at 30°
Sodium hydroxide	13·1	Less than 5
Sodium carbonate	11·4	Less than 5
Trisodium phosphate (12 H_2O)	12·0	30
Sodium sesquicarbonate	9·9	More than 60

on non-ionic detergents in acid solution, and they have both detergent and sterilising properties. They are in use in North America[6]. It is claimed that the iodine in this solubilised form has about the same killing power as the available chlorine in hypochlorites.

Chloro-compounds. The usefulness of the chlor-phenols and related compounds is well established, but they are odorous and may be irritant. Chloramine-T does not suffer from these disadvantages, but is rather mild in action. Amongst interesting new compounds are dichlorocyanuric acid and dichlorodimethyl hydantoin. It is claimed that these are of extremely low toxicity and are non-irritant.

Dichlorophene is more effective against Gram-positive organisms and fungi than hexachlorophene. Both have recently become popular as antiseptics. The latter has a phenol coefficient of about 40 against *Staph. aureus* and of about 15 against *Salm. typhi.*

Vigorous claims have been made for the efficiency and general suitability of chlorhexidine diacetate and the chloroxylenols as antiseptics. The odour of some of these substances makes them unsuitable as sterilants.

Amphoteric or ampholytic compounds. These are mostly alkylated amino acids possessing amphoteric properties, for example dodecyl β-alanine[7]. They possess many advantages according to their sponsors. Thus they are good wetting agents and fairly good emulsifiers. They are

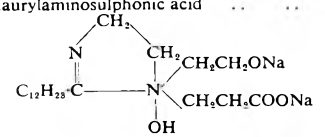
compatible with anionic, non-ionic and cationic agents. Typical examples are given in Table V.

The Importance of Specificity

Micro-organisms vary widely in their resistance to adverse conditions and to lethal agents. A good example is the difference between *N. gonorrhoea* and the spores of *B. subtilis.* The former quickly dies outside the living animal; the latter may require 1 per cent NaOH for some minutes to achieve a 99·99 per cent kill. Compared with other organisms *Myco. tuberculosis* is highly resistant to most chemicals, especially acids, and yet is rapidly killed by direct sunlight and 70 per cent ethanol. The quaternary ammonium compounds are effective against most Gram-positive bacteria but relatively feeble against the Gram-negative, and

TABLE V

AMPHOTERIC (AMPHOLYTIC) SURFACE-ACTIVE GERMICIDAL AGENTS

Chemical name or formula	Trade name	Manufacturer
Dodecyl-β-alanine	Deriphet	General Mills
Dodecyl-β-aminobutyric acid	Armeen Z	Armour
N-Laurylaminosulphonic acid	Siposan	Sipon
	Miranol	Miranol Chemical
Dodecyl-di(aminoethyl)-glycine	Tego	Th. Goldschmidt

especially *Ps. pyocyanea.* Disinfectants can be highly specific in their action, and in choosing a disinfectant consideration must always be given to this fact.

The Danger of Generalising on the Use of Germicides

Many factors influence the effectiveness of any one germicide for a given purpose. The most important are (i) concentration, (ii) temperature, (iii) time of contact, (iv) pH, (v) amount and kind of organic matter, (vi) types of organism present, and the type selected for assessment purposes, and (vii) physiological condition of organisms. Other factors of minor importance also exert some influence. For this reason it is unsound to make comparisons between disinfectants. Just as it is essential to specify techniques in detail when evaluating disinfectants in the laboratory, so it is essential to specify practical conditions of use when comparing sterilants. The rating of a disinfectant for use as a rinse solution for beer glasses, might prove to be very different when used for pipelines in a dairy. The former must be used quickly in the cold at pH 6–8; the latter can be used for some time at a high temperature at any required pH value.

The Significance of Organic Matter

The commonest cause of failure to achieve sterility, or adequate cleansing, is generally the incomplete removal of organic matter, for

example milk-stone on pasteurising plant, grease on skin, protein-fat residues on dishes. Such organic matter can interfere in two ways. As a firm film on the object it can act as a barrier between cell and germicide. If it becomes detached and goes into colloidal solution or suspension in the disinfecting liquid it completes with the cells for the disinfectant and so reduces the concentration of disinfectant. Some types, for example the quaternary ammonium compounds, may be inactivated by chemical combination with anionic material, or by adsorption on suspended matter.

The Nature of the Material

Smooth non-absorbent materials such as glass and stainless steel are easy to sterilise. Rough absorbant materials such as all animal and vegetable matter, rubber, plastics and textiles, are difficult if not impossible to sterilise. With these, sterilisation is entirely a matter of contact between sterilant and germ cell. If contact can be established by penetration (for example, of skin by 70 per cent ethanol) or soaking (for example, of blankets and napkins in a quaternary compound or in chlorine solution), then sterilisation or cleansing is quite feasible. Sterilisation may require much longer times, higher concentrations and higher temperatures.

The Nature of the Surface

One of the most important lessons which has been learned by industry and manufacturers of food equipment is the necessity for a smooth finish to, and absence of crevices and dead ends in, all items which come into contact with food, because they are difficult to free from traces of organic matter, and may escape contact with cleaning and sterilising solutions by reason of air-locks. Losses to the food industry from this cause alone must have been great in the past; fortunately the lesson has now been well learned.

Corrosion

The biggest problem in chemical sterilisation, and certainly the greatest disadvantage of chlorine, is corrosion. This may not be realised until it is too late, and damage to the extent of hundreds or thousands of pounds has been done.

Enthusiasm in sterilisation should always be tempered by a fundamental knowledge of the method. As with other developments in industry, manufacturers often acquire experience at the expense of their customers.

The fundamental aspects to consider when using hypochlorite or other corrosive sterilants are (i) to ensure a clean surface, (ii) to use the correct concentration of available chlorine (p.p.m.), (iii) to use cold or barely warm water, (iv) to use for the correct time, for example, 10 minutes, (v) to rinse *immediately* with sterile water (5 p.p.m.), and (vi) to sterilise just before use.

The practice of leaving a closed circuit system full of weak hypochlorite solution overnight is not necessarily wrong, but this must be carefully controlled. The equipment must be corrosion-resistant (for example, stainless steel or glass) and the solution must be weak, alkaline and cold.

Recontamination

Methods of sterilisation are sometimes condemned as the result of tests on the product or on the equipment, when the real cause of failure is recontamination. This is particularly true in bottle washing. Both manufacturers and users of bottle-washing machines are sometimes oblivious of the fact that although their detergent section efficiently cleans and sterilises the bottles (p. 38 *T*), these are then immediately sprayed with a warm rinse having the bacteriological properties of sewage. Although finishing with a mains water rinse it is not surprising that unsatisfactory bottles are obtained. With any machine all rinse tanks should be drained every day and the addition of a sterilant such as hypochlorite or a quaternary compound sufficient to exert a bacteriostatic effect is advantageous. Constant bacteriological control of rinse tanks is essential, and the count should never rise above 1,000 per ml. A final mains rinse should then be sufficient to ensure satisfactory bottles (p. 38 *T*). It is always sound practice to clean immediately after use, and to sterilise immediately before use.

Industrial Practice

The more perishable a food, the better it is as a medium for micro-organisms, and the more important it is to ensure sterility in the food equipment and the containers. Even if the packaged food is sterilised afterwards, a cleansed bottle or can is advantageous. The modern food technologist must *ipso facto* be a microbiologist. Apart from problems of keeping quality, high moisture foods in the pH range 6 to 8, such as milk and meat stews, are good media for the growth of pathogenic organisms, and so there is a double reason for observing the most stringent hygienic precautions in processing.

The Dairy Industry

Milk is our most perishable food and the dairy industry has attracted more legislation than any other branch of the food industry. For many years hot water and steam were the only permitted means of sterilising equipment, and rigorous requirements were laid down in the Milk and Dairies Order, 1926. During the 1939–45 war necessity brought about the introduction of new methods and the Milk and Dairies Regulations, 1949 (now 1959), permitted the use of approved sterilising solutions. The cleaning and sterilising problems in dairying are diverse and (cf. Table I) the spraying of a chemical solution is often more convenient than the use of steam. Dairy plant lends itself particularly to circulation methods of cleansing and in-place methods (see p. 38 *T*) are now being introduced. Cleaning and sterilising in the dairy industry have been revolutionised in the last 30 years and are now highly efficient and scientifically controlled processes. Chemical sterilisation is almost universal in the dairy industry today, but only approved sterilants may be used[8]. Hypochlorites and quaternary ammonium compounds are the commonest but iodophors are being introduced[9]. The ubiquitous use of chlorine, and of acids for descaling and in-place cleaning (p. 38 *T*) has

been made possible only by the now almost universal adoption of stainless steel for all dairy equipment, apart from glass and plastic for certain types of pipelines. Manufacturers have played an essential part in the revolution in sterilising methods in the dairy industry, and the highest praise must be given to them for their initiative in this respect. In the 1920's equipment was made in tinned copper and iron, and badly designed from the bacteriological aspect. Today a modern processing dairy might be described as a "hygienist's paradise". (For details of sterilising methods in the dairy industry, cf. ref.[1, 9–12]).

The Brewing Industry

The brewing industry has two important advantages over dairying. Its processes are not controlled by specific legislation, and beer may rightly be regarded as a mild antiseptic, so that the adage "beer is good for you" can be interpreted in more than one sense. Caustic soda and hot water and steam have for long been the classical cleansing agents in the industry. Brewers are extremely jealous of the organoleptic properties of their product and consequently do not countenance the introduction of anything which might affect it. Chlorine is well known to produce highly odiferous chlorphenols with traces of phenolic bodies in foods, and for this reason it is ruled out for certain branches of the food industry. Another aspect is that copper equipment is still common, and so corrosive sterilants are out of the question. Thus the brewing and dairy industries afford an interesting comparison of the influence of materials on sterilising methods in industry. There is also an interesting comparison of the influence of the product on the criteria for disinfection. Pathogens, coliforms and all lactose-fermenters, and *B. cereus* a cause of "sweet curdling" or "bitty cream" are the most feared in milk. With beer, yeasts, lactobacilli and certain streptococci or pediococci are the organisms to be destroyed. Beer has never been the cause of any infectious disease, and so pathogens are of little significance. The quaternary ammonium compounds are suitable for treating beer glasses[13].

Food Manufacture

Each branch of the food industry has its own problems. As with catering, some of these have blissfully ignored the requirements of hygiene in the past because no problem has been apparent. The Food Hygiene Regulations, 1955, should be regarded as only the start of a drive for hygiene in every branch of the food industry. For some branches of the food industry the requirements are not so severe as those for the dairy industry, so that if methods and standards adopted in the dairy industry are used a satisfactory level of hygiene should be obtained.

The Catering Industry

In the past the level of hygiene in the catering and food industries has often been low. The Food Hygiene Regulations, 1955, constitute a much needed step forward, but there are no instructions regarding sterility or to methods for sterilising food equipment. The difficulties are obvious,

and there is less need for the introduction of chemical sterilisation than in other industries. A suitable treatment for crockery, cutlery and food utensils is thorough cleaning followed by a short immersion (minimum 30 seconds) in water at about 180° F. Alternatively the use of a germicidal detergent is advisable. For hand washing, where the maximum workable temperature is 120° F., such a preparation can give crockery free from pathogens. Mops and dish cloths should be "boiled" daily.

Bottles

With reasonable care it is easy to cleanse bottles for any industry using mechanical washers. Efficient pre-rinsing and jetting (or soaking and jetting) with detergent solution at the right temperature are more important than the selection of any one detergent. The pharmacist washing bottles by hand may with advantage use a germicidal detergent as advised above for the catering industry. Recontamination (p. 36 *T*) is the greatest danger. Provided that the bottle is efficiently treated, the presence of foreign objects and impervious matter such as concrete or paint does not constitute a public health hazard[14]. A most useful and practical book on bottle washing is that by Resuggan[11].

In-place Cleaning

With rising labour costs automatic methods of cleaning and sterilising have received a good deal of attention in industry. There is little doubt that in time all cleaning and sterilising will be automatic. The two essentials are a closed circuit system and the complete absence of corrodible items. A typical sequence as used in a modern dairy is as follows.

	Minutes
Cold water rinse	5
Hot detergent circulation	10–20
Cold water rinse	5
Cold chemical sterilant or hot water circulation	15
Sterile water rinse	5

In-place methods are specially suitable for acid methods. In Europe, outside Britain, cold nitric acid 0·5 to 1 per cent is often used to clean and sterilise, and equipment such as pasteurising plant is rarely dismantled. Acid methods are not in general use in Britain (cf. ref.[9]).

Enclosed Spaces

The best way of ensuring a "clean atmosphere" is efficient air conditioning incorporating bacterial filters. Aerosol disinfectants have been accepted as the best "chemical method" of disinfecting air, but there is a natural prejudice against breathing even minute quantities of chemicals. Where a room or chamber can be sealed, formalin vapour is probably still the best, especially for efficiency and cheapness. Ethylene oxide and, in the U.S.A., β-propiolactone, have been used successfully[15]. In all vapour methods the relative humidity is an important factor. This should be at least 70 per cent.

Textiles

The sterilisation of blankets, sheets, pillow-cases, baby napkins and all similar articles is each one a special problem depending on size, thickness, and nature of material. Penetration is essential, and drastic treatment may harm or shrink the fabric. "Boiling", the classical procedure, may be undesirable because of the odours and water vapour produced, and is a cumbersome procedure for large articles. This is a field where chemical sterilisation can be used with advantage. Provided a proper procedure is adopted in hand or machine-washing effective sterilisation can be achieved[16]. Quaternary ammonium compounds are commonly used for this purpose, and their ready adsorbability introduces special problems[17]. Surface treatment will affect the extent of adsorption. McNiel and others[18] found that vegetative cells of *B. cereus* and *Brevibacterium ammoniagenes* were very sensitive, *Staph. aureus* more variable, and *Proteus* highly resistant to a benzyl quaternary ammonium compound.

Water

The basis of public health is a clean and safe water supply, and the sterilisation of water is the mother of all developments of industrial sterilisation. Water almost inevitably comes into contact with equipment, and mains water is usually of very low bacterial count. However, in factories water can easily become contaminated, and the residual chlorine is usually inadequate to prevent this. The bacteriological quality of all water used in a factory should be carefully controlled[19].

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DISCUSSION

The following points arose out of the DISCUSSION.

One of the difficulties of determining disinfectant activity was the heterogeneous nature of the bacterial population, the interest in which was centred only on those few organisms with high resistance. The use of a single test organism was inappropriate as a measure of the activity of a given disinfectant. A British standard, using staphylococcus as the test organism

would shortly be published. Phenol might be used to attempt to standardise the organism, using the dilution at which the disinfectant responded to the test organism and applying a fixed correction factor. Phenol was a bad standard for disinfectants with mechanism of action differing from it, but there would be no advantage in relinquishing it as standard unless it was replaced by some other method of quality control. The ability of an organism to grow in the presence of concentrations of phenol at a certain temperature depended on the nutrient concentration. There was an inverse relation between temperature and threshold concentration. Results from a study of the phenomenon of multiplication showed that the materials of the cell exudate contribute to growth. The course of a bactericidal action was not explained by either the mechanistic or the vitalistic theory of disinfection; it appeared that the important factor was the concentration of the active substance on the cell surface, but the ability to saturate a cell surface depended upon the materials present in the environment. Chloroxylenols were still acknowledged for their activity against streptococci, and they had the advantage of retaining their activity on the skin. Their activity against pseudomonads depended on the soap used in their manufacture and likewise the nature of the soap contributed the largest variation in the activity of lysol. For skin disinfectant, alcohol, iodine, the chloramines and possibly one of the quaternary compounds were suitable. The best antiseptic to add to soap was soda; any other addition—apart from some of the mercurials—gave no additional antibacterial properties. For apparatus sterilisation and gaseous disinfection, formaldehyde was still popular, but it had low penetration into woven material. Ethylene oxide was very effective. Beta-propiolactone was also effective but it had been shown to be carcinogenic. When there was any possibility of spore contamination a lysol type of preparation was preferred to a quaternary ammonium compound though bacterial spores had been known to survive in 5 per cent phenol. For the sterilisation of plastic surfaces (provided the surface was in good condition) it was best to clean thoroughly in liquid detergent, rinse, and immerse in 100 ppm available chlorine for one hour. The suitability of the terms "bacteriostat and bacteriostatic" for the 1963 B.P. was queried. Many thought that true bacteriostasis was impossible; a term "bacteriofrene"—a substance which curbed the growth of bacteria—had been proposed.

ALKALOIDS OF *VOACANGA SCHWEINFURTHII* STAPF

PART I. VOACAMINE AND VOBTUSINE

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Two alkaloids, voacamine and vobtusine, have been isolated from the stem bark of *Voacanga schweinfurthii* Stapf (Apocynaceae).

THE presence of alkaloid in plants of the genus *Voacanga* was noted by Greshoff[1] in 1890, but detailed investigations have only recently been made on several species, especially *V. africana* Stapf from which ten alkaloids have been isolated[2]. Vobtusine and voacamine were isolated from *V. africana* and *V. thouarsii* Roem and Schult (var. *obtusa* K. Schum) by Janot and Goutarel[3]. Vobtusine and voacangine were reported in *V. dregei* E. Mey by Schuler and others[4], but these alkaloids were not found by Neuss and Cone[5] who isolated a new base, dregamine. Vobtusine has also been reported[6] in the Apocynaceous plant *Callichilia subsessilis* Stapf.

Stem bark of *Voacanga schweinfurthii* was kindly supplied by Dr. D. B. Fanshawe, Division of Forest Ecology, Forest Department, Kitwe, N. Rhodesia through Mr. J. J. Lewis, of the University of Glasgow. Isolation of voacamine and vobtusine from this material was by the method of Percheron[7]. Two strongly basic fractions were chromatographed on alumina to give voacamine and vobtusine, each verified by melting point, rotation, ultra-violet and infra-red spectra, and micro-analysis.

EXPERIMENTAL

The bark (2·4 kg.), in No. 60 powder, was percolated to exhaustion (negative Meyer's Reagent) with ethanol (70 per cent, 45 l.). The first 15 l. of percolate was reduced to about 1 l. by distillation under reduced pressure at a temperature below 50°. The remaining thick dark brown, aqueous suspension was shaken with ethyl acetate (1·25 l.) in which most of the solid matter dissolved. The aqueous fraction was separated and shaken with further quantities of ethyl acetate (2 × 500 ml., 1 × 200 ml.). The bulked ethyl acetate solutions, after washing with distilled water (400 ml.), constituted Solution I.

To the partially extracted aqueous fraction above was added ethyl acetate (1 l.) and sufficient sodium carbonate to adjust to pH 9. After shaking and separating, the aqueous fraction was extracted with further quantities of ethyl acetate (1 l., 800 ml., 400 ml., and 5 × 200 ml.). The bulked ethyl acetate solution was divided, to facilitate handling, and each half washed with distilled water (400 ml.). The two ethyl acetate solutions constituted Solution II.

Solution I was extracted with acetic acid (5 per cent, 4 × 500 ml., 1 × 200 ml.) each extract being washed with the same ethyl acetate

(500 ml.). The combined acid fractions were basified with solution of ammonia (20 per cent) in the presence of ethyl acetate (1 l.). After shaking, the aqueous portion was separated and further extracted with ethyl acetate (2 × 500 ml., 1 × 200 ml.), the combined extracts being washed with distilled water (400 ml.). The ethyl acetate solution was dried (Na_2SO_4) and filtered, and the solvent removed under reduced pressure, to leave a residue of crude strong base (Residue IA, 3·66 g.).

Solution I, after acetic acid extraction, was extracted with hydrochloric acid (5 per cent, 2 × 500 ml., 6 × 200 ml.), the acid fractions washed with ethyl acetate (500 ml.), bulked, basified with solution of ammonia (20 per cent) and extracted with ethyl acetate (1 l., 2 × 500 ml., 1 × 200 ml.). The combined extract, after washing with distilled water (400 ml.), was dried (Na_2SO_4), filtered, and evaporated to dryness under reduced pressure to leave a residue of crude weak base (Residue IB, 0·73 g.).

Each half of Solution II was subjected to the extraction procedure described for Solution I, using different volumes of solvent. Acetic acid (5 per cent, 500 ml., 7 × 200 ml.) followed, after basifying, by ethyl acetate (500 ml., 3 × 250 ml.) gave a residue of crude strong base (Total Residue IIA, 12·22 g.). Hydrochloric acid (5 per cent, 500 ml., 8 × 250 ml.) followed, after basifying, by ethyl acetate (500 ml., 250 ml.) gave a small residue of crude weak base (Total Residue IIB, 1·51 g.).

The remaining 30 l. of initial percolate, extracted as above, gave the following yields of crude base: IA, 1·46 g.; IB, 0·72 g.; IIA, 0·70 g.: IIB, 0·02 g.

Purification of Strong Bases

The dried crude strong bases from the first 15 l. of percolate were shaken with warm benzene (Residue IA, 25 ml.; Residue IIA, 75 ml.) and after cooling the small amount of insoluble material was filtered off and the filtrates evaporated to dryness under reduced pressure. The residues were redissolved in benzene (IA, 15 ml., IIA, 50 ml.) and the solutions chromatographed on alumina (Merck) using 100 g. and 360 g. of adsorbent for residues IA and IIA respectively.

Development and elution of each chromatogram began with benzene, followed by ether, ether : methanol, and methanol. The various fractions of eluant were evaporated to dryness, the residues weighed (Table I) and attempts made to crystallise each from dry methanol.

The residues from the second fraction from each column gave, after several re-crystallisations from methanol and drying *in vacuo* over phosphorus pentoxide, white acicular crystals of voacamine (from IA, 0·06 g.; from IIA, 0·91 g.).

The residues from the third fraction from each column were shaken with methanol, the insoluble material filtered off and dissolved in methylene chloride. On addition of methanol to this solution and warming, white crystals of vobtusine were obtained and these were purified by repeating the above process. (Yield from IA, 0·10 g.; from IIA, 1·25 g.)

Voacamine. Recrystallised from methanol, corrected m.p. 223° (decomp.) readily soluble in chloroform, sparingly soluble in methanol. $[\alpha]_D^{20} = -50°$ (c = 1 in chloroform) ultra-violet spectrum (absolute ethanol) with absorption peaks at 225 mμ (log ϵ = 4·72) and 295 mμ (log ϵ = 4·28) and infra-red spectrum with absorption bands at 3,380, 2,900, 1,725, 1,710, 1,460, 1,370, and 736 cm.$^{-1}$, agreed with published[7] results. (Found: C, 71·00; H, 7·5; N, 7·8, $C_{45}H_{56}O_6N_4$ requires C, 72·3; H, 7·6; N, 7·8 per cent.)

Vobtusine. Precipitated from methylene chloride, uncorrected m.p. 305° (decomp.), readily soluble in chloroform, insoluble in methanol, deep blue colour with concentrated nitric acid. $[\alpha]_D^{20} = -295°$ (c = 1 in chloroform). Ultra-violet spectrum (absolute ethanol) with absorption peaks at 220, 265, 300, and 325 mμ agreed with results of Janot and Goutarel[3] and infra-red spectrum with absorption bands at 3,340, 1,680, 1,610, 772, 746, 732, corresponded to published[4,6] spectra. (Found: C, 70·3; H, 7·1; N, 7·65. $C_{42}H_{48}O_6N_4$ requires C, 71·6; H, 6·9; N, 7·95 per cent.) The $[\alpha]_D^{20}$ value, although lower than the published[3] figure (− 321°), was the same as that obtained by us on a genuine sample of vobtusine. The slightly low analysis is accounted for by the method of drying which has an effect on the results[6]. Our sample was dried (P_2O_5) *in vacuo* at 55° for 4 hours and at 20° for 24 hours.

TABLE I

WEIGHT OF ALKALOID REMOVED FROM CHROMATOGRAMS OF CRUDE BASE BY VARIOUS SOLVENTS

Material	Fraction	Eluant	Volume (ml.)	wt. (g.)
Residue IA	1	benzene	600	0·314
	2	ether	800	0·736
	3	ether/methanol (1 per cent)	800	1·014
	4	ether/methanol (2 per cent)	400	0·230
	5	ether/methanol (5 per cent)	400	0·166
	6	ether/methanol (10 per cent)	300	0·123
	7	methanol	100	0·167
Residue IIA	1	benzene	900	0·045
	2	ether	3,000	2·192
	3	ether/methanol (1 per cent)	3,000	2·701
	4	ether/methanol (5 per cent)	1,200	1·241
	5	methanol	300	1·121

The authors wish to thank the Trustees of the Bellahouston Fund and Professor M. M. Janot, Galenical Pharmacy Laboratory, Faculty of Pharmacy, University of Paris through whose kindness one of us (F.N.) was able to work for a short period in Paris. Also Miss M. Buchanan and Mr. W. McCorkindale for microanalyses.

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After Mr. Newcombe presented the paper there was a DISCUSSION. The following points were made.

Voacamine and vobtusine were the main alkaloids of *V. schweinfurthii* Stapf. Vocamine was known to have a 3-methoxy di- indole structure, and vobtusine was also a double indole structure with methoxy groups. Neither had been tested pharmacologically and the claimed cardiotonic activity of the alkaloids of *V. africana* had not been confirmed by others.

VEGETABLE PURGATIVES CONTAINING ANTHRACENE DERIVATIVES

PART XI. FURTHER WORK ON THE ALOIN-LIKE SUBSTANCE OF *Rhamnus purshiana* DC.

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The aloin-like substance formerly referred to as Compound A_1, has been resolved into four closely allied anthraquinone derivatives by paper chromatographic and countercurrent techniques. Two of these substances have been isolated in pure form and their general properties, melting point, optical rotation, R_F values and ultra-violet light absorption curves are recorded. Treatment with ferric chloride yields aloe-emodin from both, and each on mild hydrolysis, produces barbaloin. The names Cascaroside A and Cascaroside B are proposed. Preliminary work on the other two components has shown that they are based on a compound similar to barbaloin but a derivative of chrysophanol instead of aloe-emodin; the name chrysaloin is suggested for this substance.

IT was shown previously[1] that cascara bark (*Rhamnus purshiana* bark) contains an aloin-like substance which was named Compound A. Although this substance always gave one spot on the paper chromatographic system used, we were not satisfied it was a pure substance, mainly because of the uncertain melting point and the fact that the extinction values at the peaks of the ultra-violet light absorption curve varied from batch to batch. The proportion of aloe-emodin and chrysophanol produced by ferric chloride treatment varies from batch to batch, an observation which is also consistent with the supposition that Compound A is a mixture.

EXPERIMENTAL

Experience had shown that prolonged exposure to solvent systems containing acids led to changes in the compounds. Thus the methods of Adamis and Pawlaczyk[2] for *Rhamnus frangula* and Awe, Auterhoff and Wachsmuth-Melm[3] for aloe were attempted but not used extensively because of the presence of acetic acid. Also, the separation effected was not much better than that occurring when our previous systems were used.

Paper Chromatographic Systems

Belaart[4] used the system *n*-butanol : ethanol : water (5 : 1 : 4) for investigating *Rheum* species. Using this system, the upper layer as running solvent and Whatman No. 1 paper, by ascending technique, the original Compound A showed three spots on development. The upper one ($R_F = 0{\cdot}43$) on elution and ferric chloride treatment[1] yielded chrysophanol only: the two lower ones ($R_F = 0{\cdot}31$ and $0{\cdot}25$) both yielded aloe-emodin only.

Further experimenting led to the discovery of another useful system; ethylmethyl ketone : methanol : water (20 : 1 : 5). When used in the same conditions as the previous one the original Compound A was resolved into two upper spots ($R_F = 0{\cdot}50$ and $0{\cdot}44$) both yielding chrysophanol only

on ferric chloride treatment and two lower spots ($R_F = 0{\cdot}32$ and $0{\cdot}23$) both yielding aloe-emodin only on ferric chloride treatment. Subsequent work, to be described later, showed that the two substances with low R_F value both yielded barbaloin on mild hydrolysis. In similar circumstances the two substances of higher R_F value yielded a compound corresponding to barbaloin but based on chrysophanol instead of aloe-emodin. We propose to call this second aloin-like substance *chrysaloin*. Baumgartner and Leupin[5] state they have isolated barbaloin from cascara bark; we have confirmed this and have shown that chrysaloin also occurs as such in the bark.

To confirm that these four compounds were single substances we decided to apply countercurrent methods.

Countercurrent Systems

Since we had to work with compounds whose physical and chemical properties were largely unknown it was necessary to work on empirical lines in devising suitable solvent systems. Our previous experience with the use of selective solvents and paper chromatographic systems led us to choose the following for preliminary investigation; acetone, benzene, ethanol, ether, ethyl acetate, isopropanol, methanol and water. Various mixtures containing two or three of these solvents were prepared so that two layers were formed, the volumes of the two layers were not too dissimilar, and, on shaking and allowing to settle the layers separated readily. Mixtures which passed these tests were then used on strip chromatograms in boiling tubes. Those systems which resolved Compound A into two to four spots (none resolved it into more than four spots) were then selected for further testing in a small hand-operated countercurrent machine containing Craig tubes. On this basis the following three systems were discovered. Ether : isopropanol : water (2 : 1 : 2), Ethylmethyl ketone : water (4 : 3), n-butanol : ethanol : water (5 : 1 : 4). Two of the successful systems are almost similar to those used for paper chromatographic work.

This additional work did not reveal any further resolution of Compound A into more than four anthraquinone compounds but it did show that certain compounds fluorescing blue in ultra-violet light had R_F values identical with those of some of the four compounds. Furthermore, when an extract of the bark was used as starting material, a large number of anthraquinone compounds was revealed, some of which had R_F values similar to the compounds we were extracting. These were all successfully separated.

Use of Selective Solvents

Much of the previous work[1] was confirmed but in addition it was shown that ethyl acetate was useful in separating free anthraquinones, barbaloin, chrysaloin and other impurities from the fractions rich in "Compound A."

Isolation of Two New Compounds

A methanolic extract of the bark was prepared as previously described[1]. 100 g. of this extract was dissolved in 250 ml. of methanol and slowly

poured, with vigorous stirring, into 5 litres of ethyl acetate. The ochre-coloured precipitate, weighing about 80 g., was collected, dried, and extracted with four successive quantities of 800 ml. of boiling isopropanol. Each hot extract was filtered immediately, the filtrates combined and allowed to cool in a refrigerator. The yellow precipitate (about 40 g.) was collected, dissolved in 400 ml. water and exhaustively extracted with ethyl acetate in a continuous liquid:liquid extractor. This process removed practically all the barbaloin, chrysaloin, and certain emodin glycosides from the aqueous solution. The latter was evaporated to dryness at a low temperature in a rotating vacuum evaporator, leaving a yellow residue weighing about 30 g. Batches of 15 g. of this residue were dissolved in 28 ml. of the lower phase of the system ethylmethyl ketone: water (4:3), making a volume of about 36 ml. 18 ml. each were introduced into the first two tubes of an automatic countercurrent apparatus. Using 20 ml. portions of upper and lower layers of the above solvent system sixty transfers were effected. Paper chromatographic examination of each of the resulting 120 layers showed that the barbaloin and chrysaloin compounds were contained in the lower aqueous layers as follows. Tube 1, brown pigments mainly; tubes 2 to 5, brown pigments plus the bulk of the barbaloin compounds and some chrysaloin compounds; tubes 6 to 9, mainly chrysaloin compounds with traces of barbaloin compounds; tubes 10 to 16, mainly chrysaloin compounds with traces of certain compounds fluorescing blue in ultra-violet light. Other anthraquinone compounds were distributed elsewhere and suitable layers were retained for future work. The contents of tubes 2 to 5 (lower layers) were combined and evaporated to dryness and the residue (about 6 g.) dissolved in 24 ml. of the lower phase of the system *n*-butanol: ethanol: water (5:1:4). This solution was treated in the automatic countercurrent apparatus as before, using 20 ml. portions of the upper and lower phases of the solvent system just mentioned, and thirty transfers. Paper chromatographic examination of each of the layers showed that the barbaloin compounds had been separated from traces of chrysaloin compounds. The contents of the appropriate tubes containing barbaloin compounds were evaporated to dryness *in vacuo* and the solid residue extracted with hot isopropanol. On cooling a buff yellow precipitate was formed. This was separated, dried and dissolved in about 10 ml. of methanol and the solution slowly poured into 8 volumes of ethylmethyl ketone. After allowing the precipitate to settle the supernatant liquid was decanted, the precipitate washed with ethylmethyl ketone and allowed to dry in a vacuum desiccator. This precipitate contained only the two barbaloin compounds referred to earlier. These two were separated by band chromatography, using the ethylmethyl ketone system, and purified by elution with methanol and precipitation from ethylmethyl ketone as already described.

The entire process was repeated so that two independent batches of the barbaloin compounds were prepared. A third batch was prepared from a sample of Compound A prepared in 1958 by the method previously described[1]. The properties of the three batches were sufficiently

consistent to conclude that the two barbaloin compounds were pure substances and we propose to call them *Cascaroside A* and *Cascaroside B*.

Properties of Cascaroside A and B

Some of the physical and chemical properties of the two cascarosides are shown in Table I, and the ultra-violet light curve of cascaroside A is shown in Figure 1.

Mild Hydrolysis

Both cascaroside A and B, on heating in N hydrochloric acid at 70° for 2 hours broke down into barbaloin. This was proved by comparison

TABLE I

PROPERTIES OF CASCAROSIDES A AND B

(Note: The letters (*a*) (*b*) and (*c*) refer to separate batches of these compounds)

Properties	Cascaroside A	Cascaroside B
1. General	Buff-coloured powder; taste, sweet followed by a slight bitterness. Very soluble in water: Soluble in methanol, ethanol. Almost insoluble in acetone, chloroform and ether.	
2. R_F values n-Butanol system Ethylmethyl ketone system	0·31 0·32	0·25 0·23
3. Ultra-violet light absorption curves Peaks (mμ) E(1 per cent, 1 cm.)	267 : 295 : 323·5 (*a*) 112 157 135 (*b*) (i) 108 153 131 (*c*) 118 163 141	267 : 294 : 326 (*a*) 106 146 129 (*b*) (i) 113 157 138 (*c*) 112 158 141
4. Melting point Kofler	(*a*) 180–181° (*b*) (i) 168–169° *(ii) (*c*) 189–190°	(*a*) 165–167° (*b*) (i) 165–167° *(ii) (*c*) 181–182°
5. Optical rotation $[\alpha]_D^{20}$	(*a*) −40° (C=0·8 Ethanol) (*b*) (i) −28° (C=1·7 Water)	(*a*) −110° (C=0·8 Ethanol) (*b*) (ii) −76° (C=0·9 Water)

* After further purification by re-chromatographing and re-precipitating.

with authentic barbaloin using paper chromatographic examination, ultra-violet light curves, melting point of the isolated hydrolytic product and general physical and chemical properties. The following two procedures were used. 1. About 60 mg. of cascaroside was dissolved in 10 ml. N hydrochloric acid and kept at 70° for 2 hours. The solution was cooled and extracted with three portions of carbon tetrachloride which were rejected. The aqueous phase was then extracted with 20, 10, 10 and 10 ml. portions of ethyl acetate, the ethyl acetate extracts combined and stored over anhydrous sodium sulphate and then filtered. The filtrate was evaporated to dryness at low temperature and the brownish yellow residue stored in a vacuum desiccator (aluminium oxide) overnight. The dry residue was dissolved in 0·4 ml. warm anhydrous methanol and 1·5 ml. of carbon tetrachloride slowly added, warming the solution to redissolve any precipitate that formed. The slightly opalescent solution was allowed to cool slowly when fine yellow crystals were formed. Microscopic examination showed them to consist of well formed needles of low birefringence,

which were identical with those of barbaloin prepared in a similar manner. The melting point (Kofler) was 143° (barbaloin, 148°). The crystals were also used for paper chromatographic examination and ultra-violet light absorption curves as described in the next paragraph. 2. About 20 mg. cascaroside, dried *in vacuo* (magnesium perchlorate) at 80–100° to constant weight, was dissolved in about 10 ml. N hydrochloric acid and heated at 70° for 2 hours. The solution was cooled and made up to volume. Paper chromatographic examination showed the presence of a major spot having the same R_F value and other characteristics as pure barbaloin. The two paper chromatographic systems already referred to

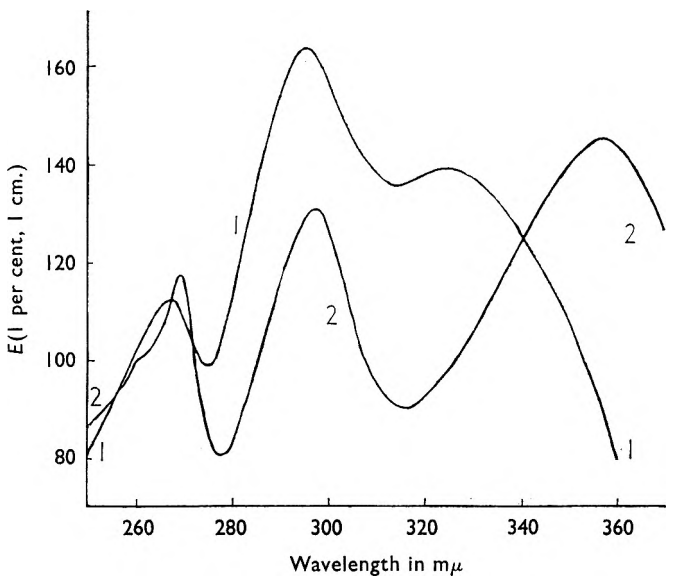

FIG. 1. Ultra-violet light absorption spectra.

1. Cascaroside A.
2. Cascaroside A after hydrolysis in N HCl at 70°.

were used and also water : acetone : benzene (2 : 1 : 4) and n-propanol : ethyl acetate : water (6 : 1 : 3). In all four systems the major spot behaved exactly as a sample of pure barbaloin prepared by the method of Hay and Haynes.[7] Traces of other anthraquinone compounds were seen but these had the same R_F values and characteristics as traces of impurities formed when pure barbaloin was treated with N hydrochloric acid in identical conditions. Spectrophotometric examination of the treated barbaloin showed that there had been no significant alteration in the E(1 per cent, 1 cm.) values taken at the peaks of the ultra-violet light absorption curve. This indicates that the amount of impurities formed is insignificant. The ultra-violet light absorption curve of the hydrolysed cascaroside was therefore determined and compared with that before hydrolysis (See Fig. 1). It will be seen that hydrolysis has produced significant changes and that the new curve has peaks exactly the same as those of barbaloin,

namely at 269, 296 and 354 mμ. The amount of barbaloin in the hydrolysate was calculated using the E(1 per cent, 1 cm.) values given by Lister and Pride[11] and from these figures the amount of barbaloin produced by 1 g. of cascaroside was calculated. The following figures were obtained in two experiments.

	269 mμ	296 mμ	354 mμ
(*a*) 1st experiment	0·550 g.	0·547 g.	0·547 g.
(*b*) 2nd experiment	0·549 g.	0·544 g.	0·559 g.

These results also show that the ratio of the peaks of the curve of the hydrolysed product is identical with that for pure barbaloin.

Discussion

The resolution of our original Compound A into two major components, one based on barbaloin (a derivative of aloe-emodin) and the other on chrysaloin (a derivative of chrysophanol) explains the fact previously reported that Compound A on treatment with ferric chloride yields aloe-emodin and chrysophanol. We have found that these major components vary in solubility in different solvents and this would explain why different batches of Compound A yielded varying proportions of aloe-emodin and chrysophanol.

Each of the major components appeared to consist of two substances and the properties of those from the barbaloin component are shown in Table I. The two substances are clearly very similar and it is probable that they are isomers. The evidence that they are two entities is as follows.

Behaviour in four paper chromatographic systems. At every stage in the extraction procedure, the fractions always showed the same two spots each with characteristic R_F values and slightly different shades of ochre to brownish red in ultra-violet light using the two chromatographic systems already referred to. Two other systems were also used to confirm the results; n-butanol : acetic acid : water (4 : 1 : 5), and n-propanol : ethyl acetate : water (6 : 1 : 3). In both additional systems two spots always appeared. When isolated in pure form and chromatographed separately or as mixtures the same two spots always appeared. When one substance contained traces of the other, there was always one large spot with a small faint one, corresponding to the proportions present.

Melting point. Each of the three batches mentioned in Table I varied in purity as judged by the melting point, but in all instances the melting point of Cascaroside A was about 10° higher than that of Cascaroside B.

Optical rotation. This is the most decisive evidence although the determination in ethanol was a little uncertain because of a darkening of the compounds when dissolved. However, both sets of figures for optical rotation show a marked difference between A and B.

Cascarosides A and B yield barbaloin on mild hydrolysis. Such a breakdown is likely to occur in the crude drug and its extracts and would explain the reported isolation of barbaloin from cascara[5]. Barbaloin has been shown to consist of aloe-emodin anthrone linked to a sugar group

by a direct carbon to carbon link[6,7]. It was the first -C-C-glycosyl compound to be found in nature and several others have since been reported[8,9]. In cascara a further four seem to be present and future research may well show that such compounds are quite common. As they are not true glycosides, yet consist of a sugar group plus an aglycone, some distinctive name should be devised for this type of natural compound.

The techniques described in this paper have revealed the presence of a number of anthraquinone compounds in cascara. Investigations to date suggest that they fall into three series, those based on aloe-emodin; mainly -C-C-glycosyl compounds; those based on chrysophanol, mainly -C-C-glycosyl compounds; and those based on emodin, mainly "easily split" glycosides.

Acknowledgements. We would like to thank Dr. D. W. Mathieson for help in determining the optical rotations and for advice on certain aspects of the work. This work forms part of a thesis to be presented by one of us (S.S.) for the Ph.D. degree of the University of London.

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After Mr. Simic presented the paper there was a DISCUSSION. The following points were made.

The cascarosides had recently been shown to be true glycosides which on hydrolysis gave the same sugar. The ultra-violet curves were obtained from solutions of pH 7. The pharmacological activity of the two compounds had yet to be assessed.

ANATOMICAL STUDIES IN THE GENUS DIGITALIS

PART II. THE ANATOMY OF THE INFLORESCENCE OF *D. lanata* L.

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The morphology and detailed anatomy of the inflorescence of *Digitalis lanata* have been described. The diagnostic characters which are the most valuable in identifying the inflorescence in admixture with the leaf are the abundance of glandular trichomes; the pollen grains; the sclerenchymatous fibres and lignified pith of the pedicel and stem; also the lignified cells of the anther, fruit wall and seed coat. Those characters which may be used to distinguish the inflorescence from that of *D. purpurea* are noted.

WE have previously described the morphology and detailed anatomy of the inflorescence of *Digitalis purpurea* L[1]. This communication describes the morphology and anatomical structure of *D. lanata* L.

The morphological description is complementary to the descriptions already made[2–6].

Material

The inflorescences used were from several clones kindly provided by the Royal Botanical Gardens, Kew, and by the Chelsea Physic Gardens. The mature flowers possessed those characters of floral morphology accepted as typical of *Digitalis lanata* L.

Experimental Methods

These were the same as described previously[1].

GROSS MORPHOLOGY

In the mature flower the calyx consists of five deeply partite sepals. The posterior and posterior-lateral sepals are lanceolate, about 8 mm. long and 2·5 mm. broad; the anterior-lateral sepals are ovate, about 8 mm. long and 3·5 mm. broad (Fig. 1, A). The gamopetalous corolla tube is about 9 mm. long, 7 mm. wide at the distal end and 2·5 mm. wide at the proximal end. Each of the five net-veined petal lobes has a recurved margin. The anterior petal is about 15 mm. long, its large free lobe bends upwards and as a flap almost closes the corolla tube (Fig. 3, A).

The androecium consists of four didynamous stamens. The filaments are slightly pubescent, curved and about 1 mm. wide. The variation in length of the adherent portion is similar to that described for *D. purpurea*.[1] Anther lobes are slightly pubescent, about 2·5 mm. long and 1 mm. wide (Fig. 4, A). Dehiscence is introrse. The syncarpous gynaecium is about 4 mm. long and 2 mm. wide at the base. The nectary is a slightly undulating ring of tissue at the base of the ovary (Fig. 5, A). The style,

firm, erect, pubescent except near the stigma, arises from the apex of the ovary and is 3–4 mm. long and 0·5 mm. wide. The stigma, terminal and glabrous is formed of two small lobes (Fig. 5, B).

The brownish-green fruit with its erect, persistent calyx, is about 10 mm. long and 4 mm. wide at the base. Dehiscence is septicidal (Fig. 6, A and B).

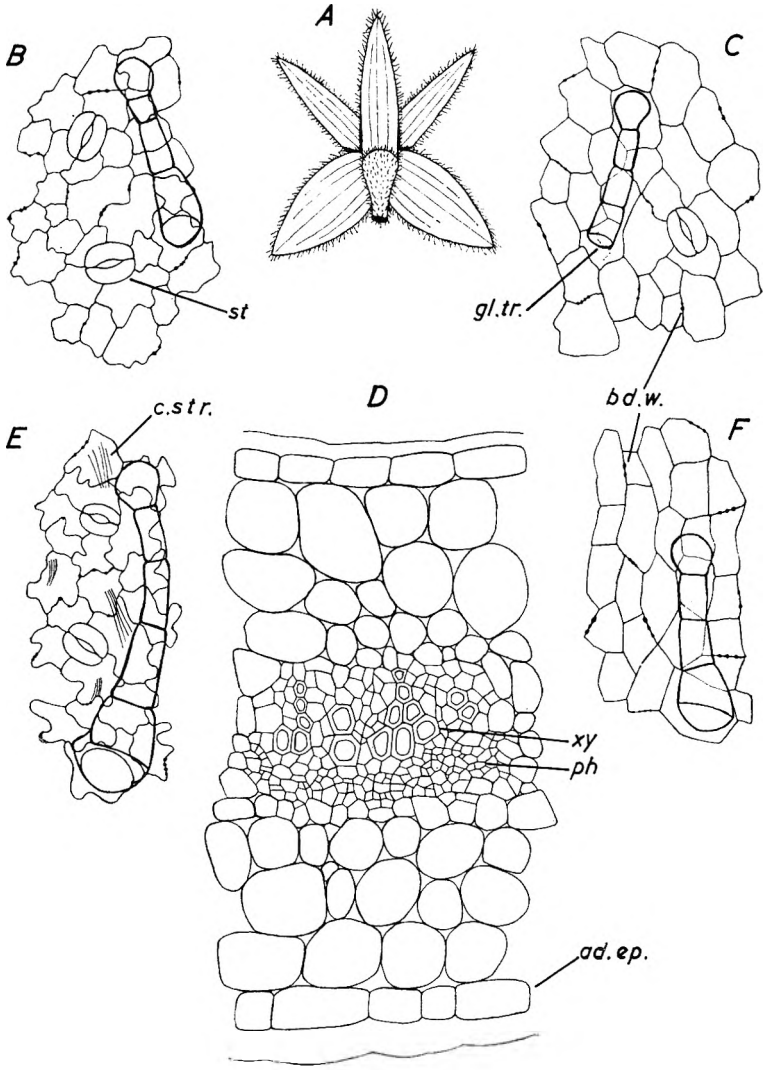

FIG. 1. *Digitalis lanata* L. Calyx. A, Ventral view of Calyx × 2. B, Abaxial epidermal cells from apical and central regions. C, Abaxial epidermal cells from basal region, both × 170. D, Transverse section through a main vein × 350. E, Adaxial epidermal cells from apical and central regions. F, Adaxial epidermal cells from basal region, both × 170. ad. ep., adaxial epidermis; bd.w., beaded walls of epidermal cells; c.str., cuticular striations; gl.tr., glandular trichome; ph, phloem; st, stoma; xy, xylem.

The numerous reddish-brown sub-conical seeds are about 1·5 mm. long and 0·75 mm. broad with at least one obvious groove indicating the line of the raphe. They vary much in shape from trapezoid to nearly ovoid. A small circular scar at one end of the groove marks the position of the hilum (Fig. 2, B).

Histology

Calyx. The abaxial surface is covered by a thick, smooth cuticle. The epidermal cells at the base are polygonal with straight or slightly

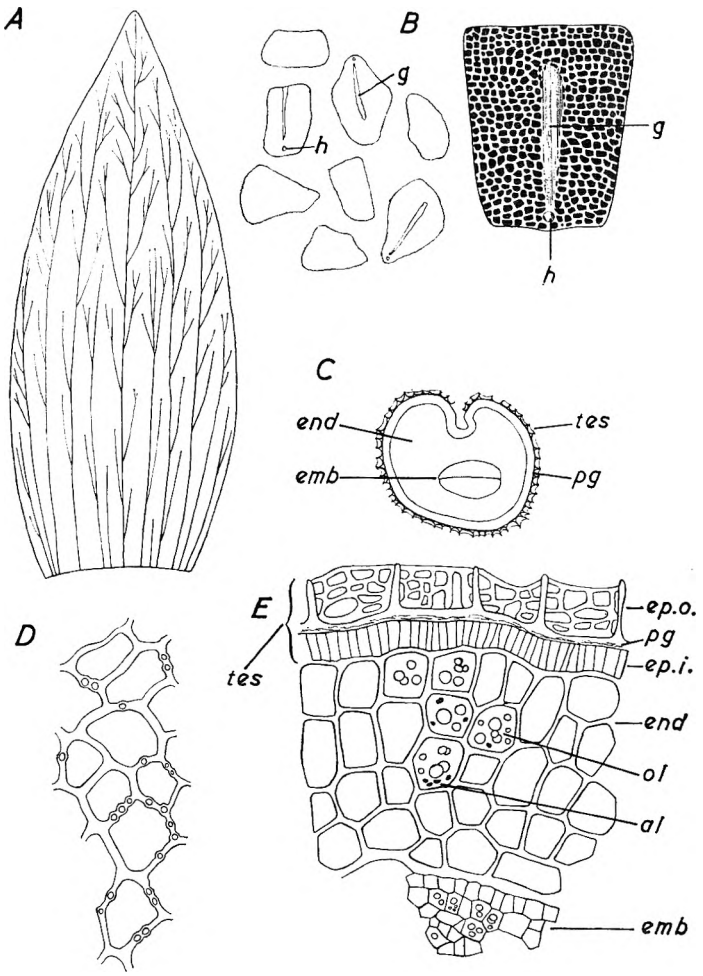

Fig. 2. *Digitalis lanata* L. Calyx and seed. A, Sepal, plan of venation × 7. B, Outline of seeds showing variation in shape × 8, also one seed × 16 showing macroscopical detail. C, Tissue plan of transverse section of seed × 16. D, Testa in surface view × 170. E, Transverse section of seed × 170. emb, embryo; end, endosperm; ep. i., inner epidermis; ep. o., outer empidermis; g, groove; h, hilum; ol, oil globules; al, aleurone grains; pg, pigment; tes, testa.

wavy beaded walls, *L and T[7] 16–**30**–**40**–60 μ (Fig. 1, C). Over the remainder of the surface the epidermal cells have similar measurements, their walls are beaded, wavy or very wavy (Fig. 1, B). There is no elongation of epidermal cells over the veins. Anomocytic[8] stomata are present on this surface (Fig. 1, B and C), they are 32–**38**–46 μ long, 20–**26**–36 μ wide. The stomatal index is not more than 1 in the basal region and up to 7 with a mean of 4 over the rest of the surface. Abundant trichomes, mainly glandular, are present. They have a unicellular head and a unicellular or multicellular pedicel (Fig. 1, B and C), on the edges of the sepals they occur in numbers varying from 27–**36**–**58**–64 per mm., they are 200 μ–**1·2** mm.–1·8 mm. long, 28–**52**–84 μ wide at the base, with 3–**6**–8 cells per pedicle. In the interneural areas there are 54–**72**–**90**–114 per sq. mm., they are 98–**350**–**400**–1400 μ long, 28–**42**–**56**–98 μ wide at the base, with 1–**3**–6 cells per trichome. Non-glandular trichomes with a smooth cuticle and an acute apical cell, occur only on the margin of the sepals near their apex. They are 240–**840**–1380 μ long 24–**36**–60 μ wide at the base, with 2–**4**–7 cells per trichome.

On the adaxial surface the epidermal cells are similar except that the cuticle is somewhat thinner and is striated in the central and apical regions (Fig. 1, D and E). Stomata also are similar, but their distribution differs, being absent in the basal region and rare over the remainder of the surface, where the stomatal index is about 3. Glandular trichomes only are present, 48–**120**–**168**–300 μ long, 12–**24**–48 μ wide at the base, 1–**3**–6 cells per pedicel. Their number per sq. mm. varies from 50–110, being least in the basal region.

The interneural mesophyll is a loose tissue of about 10 rows of undifferentiated round to ovoid parenchymatous cells containing chloroplasts with associated starch grains (Fig. 1, D). In each sepal the vascular tissue consists of about five main veins, with secondary and tertiary veinlets in the central and apical regions (Fig. 2, A). Each of these main collateral strands consists of a small roughly crescent-shaped arc of xylem vessels, about 14–30 μ in diameter, arranged in small groups or files. Medullary ray cells separate the groups or files. Adjacent to the arc of xylem is a a narrow band of phloem consisting of sieve tubes 4–10 μ in diameter, with companion cells and phloem parenchyma (Fig. 1, D). Starch sheath and sclerenchymatous fibres were not present in those sepals examined. The veinlets consist of a small group of polygonal tracheids 3–6 μ in diameter, with a small amount of indistinct phloem tissue.

Corolla. The abaxial surface is covered by a thin, smooth cuticle. Those epidermal cells around the base of the corolla tube and extending about half way along the posterior petals are polygonal, straight-walled cells, elongated in the direction of the longitudinal axis of the petals.

* In recording measurements the letters L, T and R, have reference to the longitudinal, tangential and radial directions respectively, of the plant member in question. In cases where orientation of the subject relative to the plant axis is absent, values for L and T are combined. In any set of measurements a number in bold type refers to the mean. Where most commonly occurring sizes do not appear, by inspection, to be similar to the mean, two numbers in bold type are given. These refer to the most commonly occurring size range.

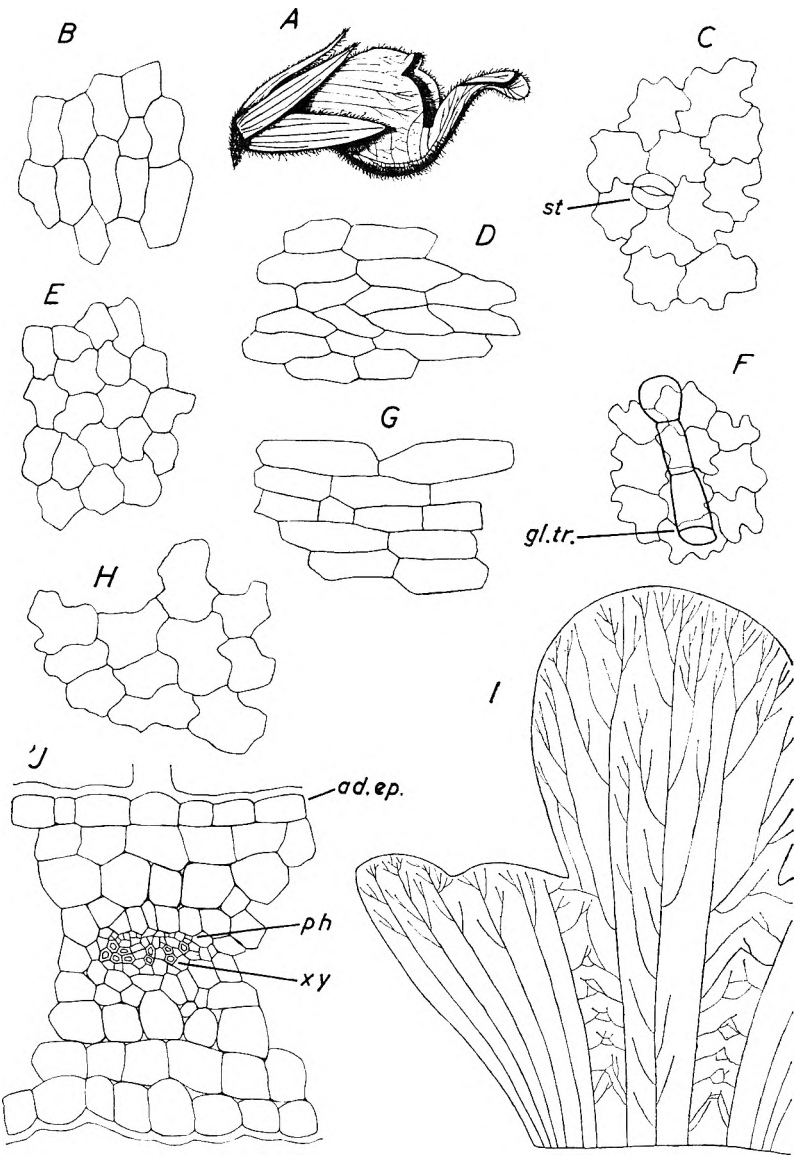

FIG. 3. *Digitalis lanata* L. Corolla. A, lateral view of corolla × 2. B, Abaxial epidermal cells from central region. C, Abaxial epidermal cells from distal region. D, Abaxial epidermal cells from proximal and posterior central region. E, Adaxial epidermal cells from central region. F, Adaxial epidermal cells from distal region. G, Adaxial epidermal cells from posterior central region. H, Adaxial epidermal cells from proximal region, all × 170. I, Corolla, plan of venation × 4. J, Transverse section through vascular bundle of corolla × 170. ad. ep., adaxial epidermis; gl. tr., glandular trichome; ph, phloem; st, stoma; xy, xylem.

They measure L, 40–**78**–120 μ, T, 12–**20**–30 μ, R, 16–**20**–28 μ (Fig. 3, D). Over the rest of the surface the cells are nearly isodiametric with wavy walls, very wavy on the free lobes (Fig. 3, B and C). They measure L and T, 24–**35**–**64**–90 μ, R, as for cells at the base of the petal. Stomata similar to those on the calyx are found on this surface (Fig. 3, C), although they are absent from the basal region and very rare on the free lobe of the anterior petal. Apart from these areas the stomatal index varies from 5–**10**–**25**–32. Trichomes similar to those found on the calyx are present, the glandular type predominating. These are more numerous in the distal region, especially on the free lobe of the anterior petal. There are 3–11 per sq. mm., they are 84–**308**–560 μ long, 14–**42**–60 μ wide at the base, with 2–**3**–5 cells per pedicel. Non-glandular trichomes are present on the margin and extreme distal area of the anterior petal. There are 2–13 per mm. of margin and 0·3–1 per sq. mm. of interneural area. They are 250–**434**–700 μ long, 28–**42**–60 μ wide at the base, with 2–**5**–7 cells per trichome.

On the adaxial surface the cuticle is smooth. The epidermal cells are polygonal and nearly isodiametric in the apical region becoming increasingly elongated in part of the central region; their outer walls are dome-shaped, (Fig. 3, E, F, G and H). The elongated cells from the posterior part of the central region measure L, 30–**68**–100 μ, T, 20–**26**–40 μ, the nearly isodiametric cells over the rest of the surface measure L and T, 16–**24**–**34**–50 μ. R for all these cells is 16–**32**–40 μ. Glandular trichomes are present on the anterior petal in numbers from 3–9 per sq. mm. On the lateral petals they are present only on the central and basal regions in numbers from 1–6 per sq. mm. On the posterior petals they are absent or rare. They measure 56–**224**–460 μ long, 14–**28**–70 μ wide at the base, with 1–4 cells per pedicel. Non-glandular trichomes are present in the apical region only in numbers from 0·3–3 trichomes per sq. mm. They are 210–**400**–700 μ long, 28–**40**–70 μ wide at the base, with 1–6 cells per trichome.

The mesophyll is similar to that of the calyx save for the absence of chloroplasts with starch grains and the presence of anthocyanin and flavone pigments and smaller intercellular air spaces (Fig. 3, J). Twelve to 18 main veins are found in the corolla together with secondary and tertiary veinlets which unite the main veins. These veinlets are particularly numerous in the areas of fusion between the anterior and lateral petals (Fig. 3, I). Veins and veinlets are similar in structure except for the size and number of the elements. The xylem vessels, 4–10 μ in diameter, with polygonal, lignocellulosic walls having spiral and annular thickening, are arranged in groups or files separated by medullary ray cells. The phloem tissue contains sieve tubes 3–6 μ in diameter, companion cells and phloem parenchyma (Fig. 3, J).

Androecium. The filament is covered by a thin, smooth cuticle. The thin walled, polygonal epidermal cells are elongated, the elongation is greatest in the central region where they measure L, 60–**96**–170 μ, T, 12–**18**–22 μ. In the apical and basal regions, L, 30–**42**–50 μ, T, 14–**18**–24 μ and L, 60–**86**–110 μ, T, 16–**28**–36 μ respectively. Throughout R

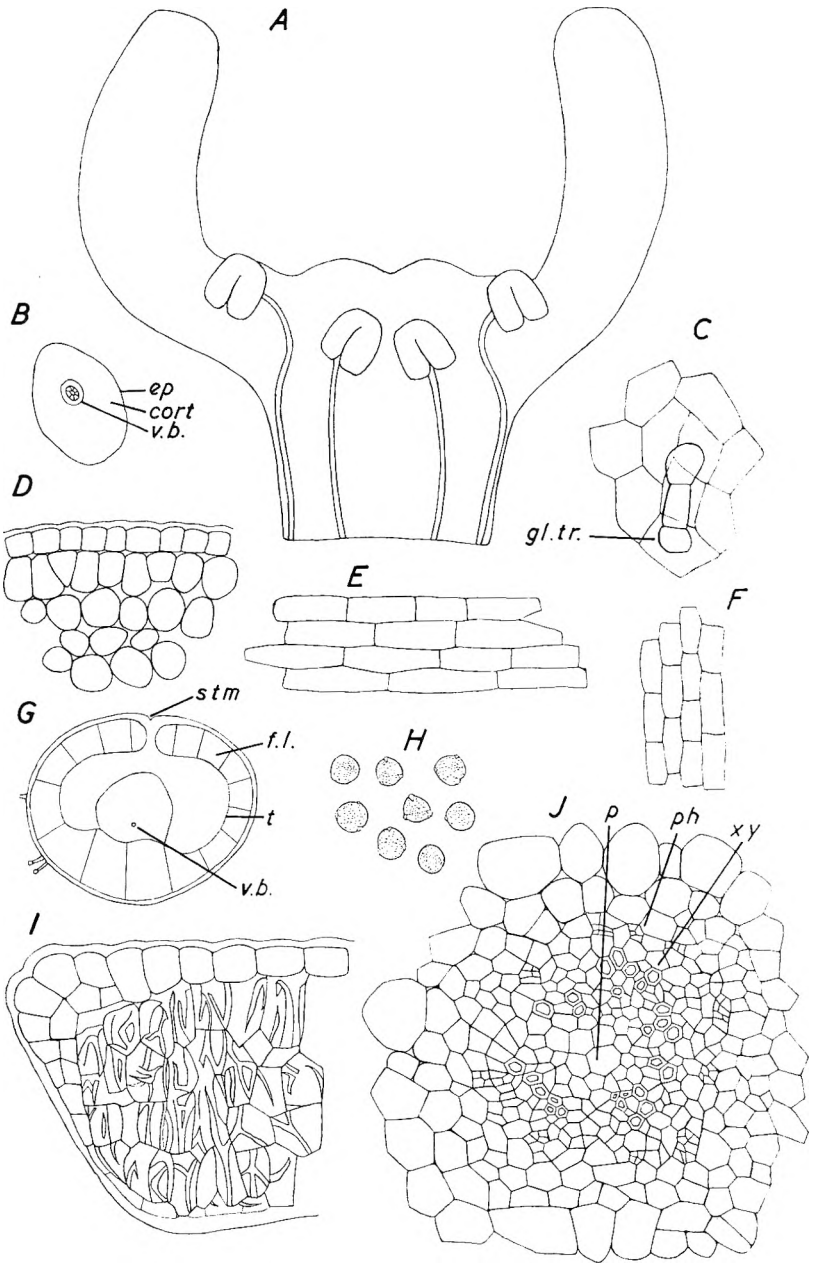

FIG. 4. *Digitalis lanata* L. Androecium. A, Plan of corolla to show position of stamens × 5. B, Tissue plan of transverse section of filament × 25. C, Epidermal cells from anther lobes. D, Transverse section of filament, epidermis and cortex E, Epidermal cells from filament. F, Epidermal cells from filament near anther, all × 170. G, Tissue plan of transverse section of anther before dehiscence × 30. H, Pollen grains. I, Transverse section of anther wall at stomium, both × 170. J, Transverse section of vascular strand of filament × 350. cort, cortex; ep, epidermis; f.l., fibrous layer; gl. tr., glandular trichome; p, pith; ph, phloem; stm, stomium; v.b. vascular bundle; t, tapetum; xy, xylem.

measures 16–20 μ (Fig. 4, D, E and F). Stomata were not found on the filaments. Glandular trichomes are present on the free portion of the filament in numbers from 8–31 per filament. They measure 70–**168**–300 μ long, 14–**28**–56 μ wide at the base, with 1–3 cells per pedicel.

The cortex consists of a loose tissue of about 5–18 rows of rounded or irregularly rounded, thin walled parenchymatous cells, with numerous intercellular air spaces, no cell inclusions were observed (Fig. 4, D). Embedded in the cortex is the slightly eccentric vascular strand. It consists of a small central pith surrounded by a discontinuous ring of polygonal, lignified xylem vessels 4–8 μ in diameter, with spiral and annular thickening. Around the xylem are groups of phloem with sieve tubes 3-5 μ in diameter (Fig. 4, J).

The anther lobes are covered by a thin cuticle. The epidermal cells are polygonal with slightly wavy walls (Fig. 4, C), L and T, 28–**38**–**52**–60 μ, R, 18–**24**–30 μ. Beneath the epidermis there are about five rows of nearly isodiametric cells with ligno-cellulosic thickening in spiral and annular bands (Fig. 4, G and I). They measure L and T, 16–**26**–**34**–50 μ, R, 20–**32**–**48**–60 μ. Remains of the collapsed tapetum are visible within this fibrous layer. Stomata are absent from the anther lobes. There are about 70 glandular trichomes on each lobe, 70–**110**–170 μ long, 14–28 μ wide, with 2–4 cells per pedicel (Fig. 4, C).

The pollen grains (Fig. 4, H), are subspherical, 20–**24**–28 μ in diameter, with three germinal pores. The intine is smooth, the exine very finely pitted. Starch and oil were not found in the grains examined.

Gynaecium. The ovary is covered by a thin smooth cuticle. The polygonal epidermal cells with straight walls measure L and T, 14–**18**–**24**–32 μ, R, 15–**20**–30 μ (Fig. 5, E). Stomata occur, length 18–**24**–28 μ, breadth 20–**22**–26 μ. Stomatal index varies up to 0·5. Glandular trichomes only occur, similar to those on the calyx, 84–**250**–**430**–980 μ long, the longest being near the apex of the gynaecium, 14–**35**–70 μ wide at the base, with 2–**3**–**4**–7 cells per pedicel. There are 0·3–**1**–2 trichomes per sq. mm. (Fig. 5, C, E and J). The cells of the inner epidermis (Fig. 5, F) are thin, straight walled and elongated at right angles to the longitudinal axis of the ovary, they measure L, 40–**46**–54 μ, T, 4–8 μ, R, 8–**10**–14 μ. Trichomes and stomata were not found on this inner surface.

The mesophyll is formed of polygonal cells with thin straight walls. They contain chloroplasts with associated small, round starch grains (Fig. 5, J). About 30 vascular strands are embedded in this mesophyll, the polygonal, lignified vessels, 4–8 μ in diameter, have spiral and annular thickening. The phloem is composed of very small elements. The placenta is formed of rounded cells with numerous intercellular air spaces (Fig. 5, I). About 10 vascular strands, similar to those in the mesophyll supply the placenta. Numerous ovules are borne on the placenta (Fig. 5, C).

The style is covered by a thin smooth cuticle. The polygonal, elongated, thin, straight walled epidermal cells measure L, 30–**46**–68 μ, T, 8–**16**–28 μ, R, 10–**12**–15 μ (Fig. 5, G). The cortex consists of round cells in which are embedded, opposite to each other and near the epidermis, two vascular

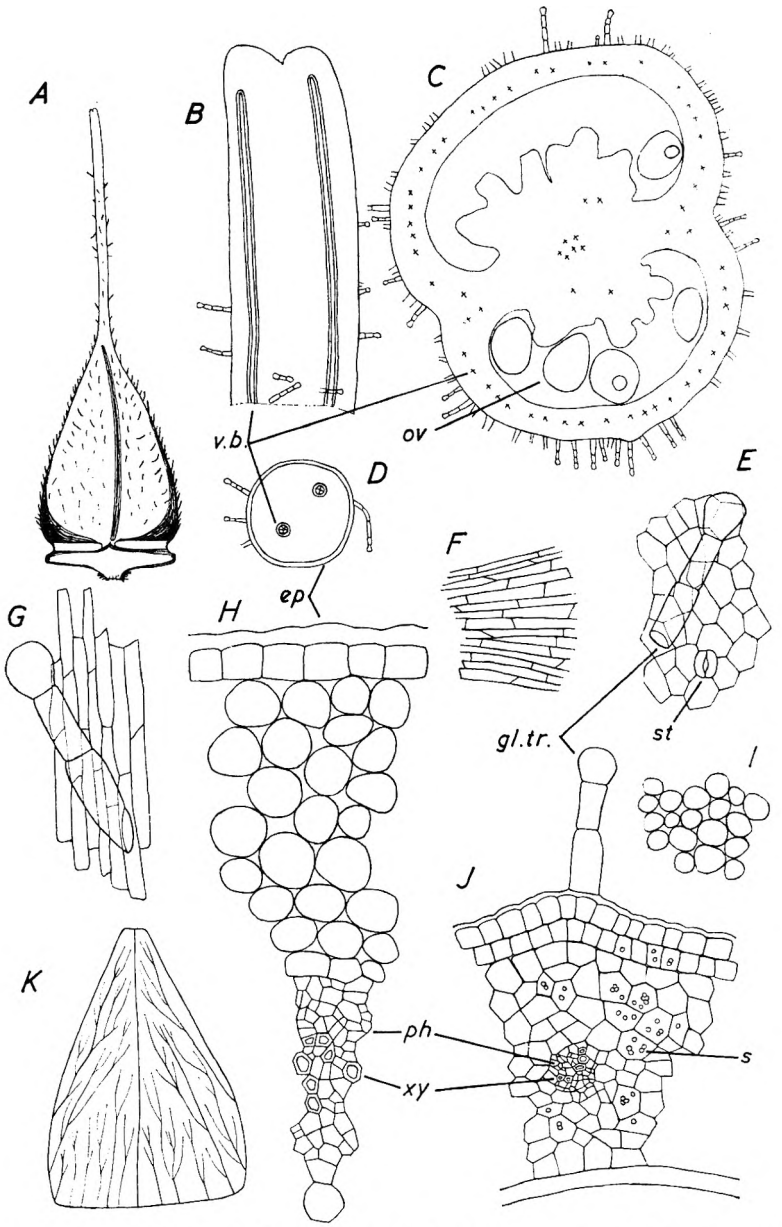

FIG. 5. *Digitalis lanata* L. Gynaecium. A, Gynaecium × 5. B, Stigma and part of style with vascular strands × 17. C, Transverse section of ovary × 17. D, Transverse section of style × 17. E, Outer epidermal cells from ovary wall. F, Inner epidermal cells from ovary wall. G, Epidermal cells from style, all × 170. H, Transverse section of style through vascular strand × 350. I, cells of placenta. J, Transverse section of ovary wall, both × 170. K, Part of ovary wall, plan of venation × 7. ep, epidermis; gl. tr., glandular trichome; ov, ovule; ph, phloem; st, stoma; v.b., vascular bundle; xy, xylem; s, starch.

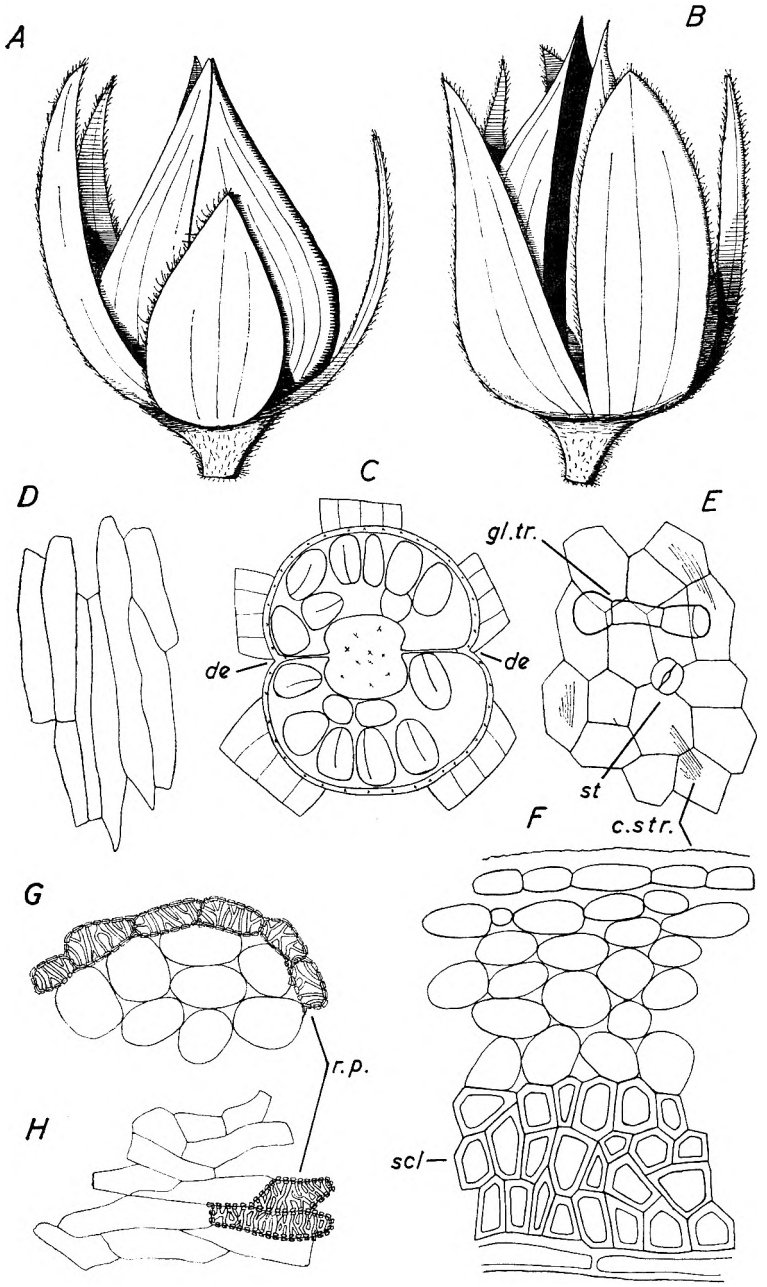

FIG. 6. *Digitalis lanata* L. Fruit. A, Fruit. B, Fruit after dehiscence. C, Transverse section of fruit, all × 5. D, inner epidermal cells of fruit wall. E, Outer epidermal cells of fruit wall. F, Transverse section of fruit wall. G, Transverse section of placenta. H, Covering cells of placenta in surface view, all × 170. c.str., cuticular striations; de, dehiscence lines; gl. tr., glandular trichome; scl, sclerenchyma; st, stoma; r.p., reticulate parenchyma.

strands (Fig. 5, B and D). Each strand (Fig. 5, H), consists of a ring of lignified, polygonal xylem vessels, 4–10 μ in diameter, surrounded by a ring of very small celled phloem. The two strands supply the two lobes of the parenchymatous stigma (Fig. 5, B). Glandular trichomes occur on the style, except near the stigma (Fig. 5, B). There are 160–230 per style, 140–**280**–630 μ long, 28–**40**–70 μ wide at the base, with 2–**3**–5 cells per pedicel.

Fruit. The outer surface is covered by a thick cuticle, sometimes striated in the central and apical regions. The straight walled polygonal epidermal cells measure L and T, 24–**28**–**60**–90 μ, R, 12–**16**–20 μ (Fig. 6, E). Stomata similar to those on the ovary are present. Glandular trichomes only occur, 2–4 per sq. mm., 112–**250**–560 μ long, 14–**40**–56 μ wide at the base, with 2–**3**–5 cells per pedicel. The cells of the inner epidermis are similar to those of the ovary except that they are larger (Fig. 6, D). Trichomes and stomata are absent from this surface.

The mesophyll is formed of about 10 rows of cells. About 4 rows adjacent to the endocarp are sclerenchymatous, the remainder parenchymatous. These latter consist of a fairly loose tissue of round or ovoid cells containing some chloroplasts. The sclerenchymatous cells have thick stratified walls without pits (Fig. 6, F), they measure L and T, 22–**26**–**54**–70 μ, R, 14–**30**–50 μ. The vascular strands are similar to those in the ovary wall. The placenta has become covered with reticulate parenchymatous cells (Fig. 6, G and H). At the fruit stage the septum is bounded on both sides by the inner epidermis and sclerenchymatous layer (Fig. 6, C). The dehiscence is septicidal, splitting occurs in the central parenchyma.

Seed. The epidermal cells of the testa are lignified, reticulately thickened on the anticlinal walls, nearly isodiametric cells with the outer wall collapsed (Fig. 2, C, D, and E), L and T, 34–**46**–**66**–120 μ, R, 30–**40**–50 μ. The reddish-brown collapsed cells beneath the epidermis form a pigment layer. Beneath these there is a single row of palisade-like cells which is the inner epidermis of the testa. The endosperm consists of straight walled polygonal cells containing oil and protein. The central dicotyledenous embryo consists of cells similar to the endosperm cells but smaller and with thinner walls, they also contain oil and protein (Fig. 2, E).

Pedicel. The surface is covered by a thick smooth cuticle. The polygonal, elongated epidermal cells have thickened walls (Fig. 7, F), L, 36–**60**–80 μ, T, 12–**16**–22 μ, R, 7–**10**–14 μ. Stomata similar to those on the calyx are present, Stomatal Index varies from 0–**3**–6. Glandular trichomes only occur, 28–**470**–840 μ long, 28–**60**–110 μ wide at the base, with 2–**4**–7 cells per pedicel, there are 16–**20**–25 per sq. mm.

The cortex consists of about 9 rows of cells, the outer 3 of collenchyma, the rest parenchyma (Fig. 7, D). Within this cortex there is a complete band of about 6 rows of sclerenchymatous fibres, 8–26 μ in diameter and about 840 μ long, with slit-like pits in their walls (Fig. 7, D and G). These fibres surround a narrow band of phloem tissue with thin walled sieve tubes 8–12 μ in. diameter. Cambial tissue is not distinct. The

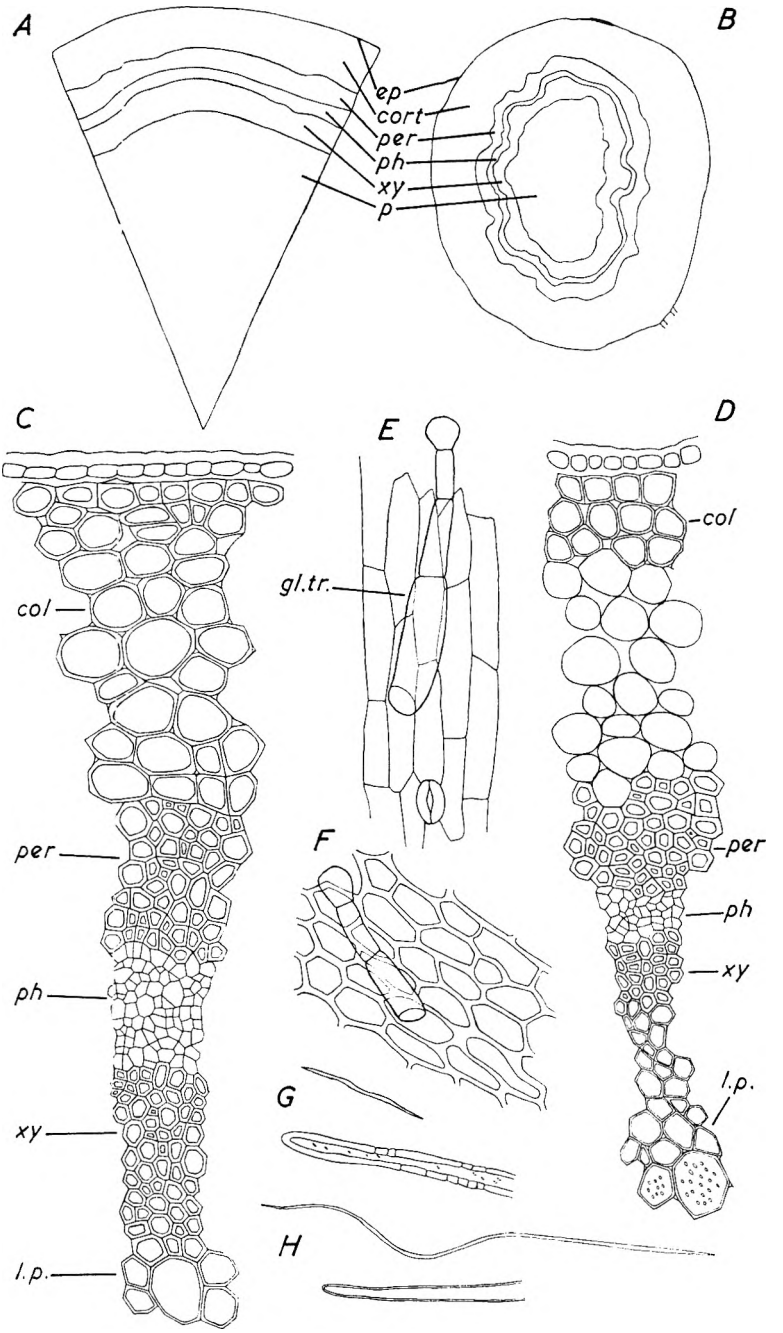

FIG. 7. *Digitalis lanata* L. Pedicel and inflorescence axis. A, Tissue plan of transverse section of inflorescence axis. B, Tissue plan of transverse section of pedicel, both × 17. C, Transverse section of inflorescence axis. D, Transverse section of pedicel. E, Epidermal cells of inflorescence axis. F, Epidermal cells of pedicel, all × 170. G Fibre from pedicel × 50, part of same × 170. H, Fibre from inflorescence axis × 50, part of same × 170. col, collenchyma; cort, cortex; ep, epidermis; gl. tr., glandular trichome; l.p., lignified pith; per, pericycle; ph, phloem; p, pith; xy, xylem.

xylem is entirely lignified, the polygonal vessels with straight and thickened walls are 8–20 μ in diameter, the thickening is annular and spiral. The central pith cells are polygonal, lignified and pitted (Fig. 7, D).

Inflorescence axis. The histology of the inflorescence axis is similar to that of the pedicel, the differences only will be noted. The epidermal cells are thin walled and larger, L, 80–**120**–160 μ, T, 14–**22**–35 μ, (Fig. 7, E). The trichomes are not so numerous, 5–**9**–11 per sq. mm. The cortex is mainly of collenchyma, although this factor varies with the age of the axis. The fibres, 8–30 μ in diameter, have thinner walls without pits and are longer—up to 2·5 mm. (Fig. 7, H). The ring of vascular tissue is nearer to the periphery of the organ in the axis than in the pedicel (Fig. 7, A and C).

Discussion

The diagnostic characters, other than the abundance of glandular trichomes, which are the most valuable in identifying the inflorescence, when in admixture with the leaf[9], are as follows.

Corolla. Epidermal cells not beaded. Corolla pigments appearing pink in chloral hydrate solution.

Androecium. Elongated epidermal cells from the filament. Epidermal cells not beaded: fibrous layer from anther wall: pollen grains.

Gynaecium. Elongated epidermal cells from style: epidermal cells not beaded: ovules.

Fruit. Sclerenchymatous cells from fruit wall: reticulate parenchyma from placenta: epidermal cells not beaded.

Seed. Lignified reticulate cells from the testa: storage tissue containing oil and protein.

Pedicel. Elongated, thickened epidermal cells: sclerenchymatous fibres: lignified, pitted pith cells.

Inflorescence axis. Elongated epidermal cells, not beaded: sclerenchymatous fibres: lignified pith cells.

This inflorescence differs from that of *D. purpurea* L.[1] in the following characters.

Calyx. Beaded epidermal cells: mainly smooth cuticle: greater Stomatal Index: Non-glandular trichomes fewer and longer: Glandular trichomes more numerous and longer: Absence of sclerenchymatous fibres and starch sheath.

Corolla. Stomatal index greater: Non-glandular trichomes fewer and shorter.

Androecium. Glandular trichomes present on filament and anther lobes.

Gynaecium. Stomatal index less: fewer trichomes: glandular trichomes present on style.

Fruit. Striated cuticle: fewer trichomes: sclerenchymatous cells neither pitted nor U-shaped.

Pedicel. Thickened epidermal cells: smooth cuticle: glandular trichomes fewer and longer: non-glandular trichomes absent: pith cells pitted and lignified.

Inflorescence axis. Trichomes as on the pedicel: pith cells not pitted. When the inflorescences of *D. lanata* and *D. purpurea* occur together in a powder the former may be identified by means of the following characters: beaded epidermal cells: pubescent androecium and style: regularly thickened, non-pitted sclerenchymatous cells of the fruit wall.

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After Mr. Cowley presented the paper there was a DISCUSSION.

STUDIES IN THE GENUS DIGITALIS

PART VI. VARIATIONS IN GLYCOSIDAL CONTENT OF BRITISH CLONES OF *Digitalis purpurea*

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Seeds of wild *Digitalis purpurea* from 150 different areas in 20 counties of Great Britain have been collected; weight of 100 seeds was 3·5–**8·6**–11·6 mg. Each batch of seed, regarded as a clone, was grown under uniform conditions and leaf collected from both first year and second year plants. Glycosidal content of each leaf sample, drawn from 8 plants within the clone, was estimated using 3,5-dinitrobenzoic acid and results expressed as u./g. by comparison with the Standard Preparation of Prepared Digitalis. Values for clones were: first year 9·8–**13·7**–18·9 u./g.; second year 3·1–**7·6**–11·4 u./g. Clone values for dried leaf yielded per plant were: first year 38–**79**–137 g.; second year 12–**23**–47 g. The weight of parent seed does not affect the yield of leaf or its activity (first year); nor is leaf activity affected by leaf yield (first year). A positive relation existed for first and second year leaf activity values for each clone and suggested genetical control of activity.

VARIATIONS in potency of different samples of dried leaves of *Digitalis purpurea* estimated by biological assay have been reported by various workers. Wokes[1] examined eight commercial samples of English leaves and found variations of 64–148 per cent from average. Watson and James[2] collected leaf and seed samples from 16 different plants in England and Wales and concluded that the potency variation 5·5–**12·4**–21·2 u./g. was not related to environmental factors of soil or altitude. The first and second year plants from these collected seeds were examined for genetical factors controlling potency[3,4]. Mather and Dyer[5] examined six strains of plants from wild and cultivated parents and concluded that heritable differences in activity and in yield of leaf existed between strains within the species. Barnard and Finnemore[6] selected one variety of seed because of the high potency of its progeny. More recently van Os and collaborators[7] have studied the heredity of proportionality between different glycosides in the total glycosidal complex for different lines of *D. purpurea*, using chemical methods of estimation.

Investigations of the natural variation within *D. purpurea* and of the possible existence of genetically controlled strains of high therapeutic potencies have been limited by the biological assays involved. The modern use of colorimetric methods of estimation of digitalis glycosides makes possible a much larger survey. The present work was undertaken to investigate the range of activity found in British samples of *D. purpurea* using a chemical method of estimation and with a view to subsequent examination of the heritability of high or low activities.

Collections

Each seed sample was collected from a few fully matured capsules on one inflorescence axis of a single plant. At the same time an objective assessment of the plant (A+ to C) was recorded together with its exact location and its environment. A distance of not less than one mile separated each plant from which collection was made and thus it was hoped that each sample of seeds might represent a separate clone of plants. Collections of 150 samples were made in September, 1950, from 20 counties in England, Wales and Scotland. These counties and the numbers of samples therefrom are recorded in the first two columns of Table I. Some predominance of samples from North Wales was arranged

TABLE I
GEOGRAPHICAL SOURCES OF SEEDS

County	Number of seed samples collected	Range of activity of progeny (first year leaf) u./g.*
Buckinghamshire	1	14·5
Cheshire	3	12·0–13·1–13·9
Cornwall	7	10·0–12·4–15·0
Cumberland	7	13·8–15·1–16·6
Derbyshire	4	12·3–13·9–15·6
Devonshire	1	13·6
Co. Durham	13	12·4–14·6–17·9
Kent	5	12·4–13·0–14·4
Shropshire	2	12·9–14·4–15·8
Staffordshire	10	11·5–13·9–15·6
Sussex	2	11·5–13·3–15·0
Westmorland	6	13·2–14·3–14·8
Anglesey	3	11·7–12·8–14·9
Caenarvonshire	32	9·8–13·1–18·6
Denbighshire	21	11·9–14·3–18·9
Merionethshire	1	13·3
Aberdeenshire	19	11·5–13·9–16·3
Dumfriesshire	11	11·3–13·2–15·4
Lanarkshire	1	11·3
Stirlingshire	1	No germination
Total	150	

* Total glycosides were estimated by the 3,5-dinitrobenzoic acid process and the results expressed as units per gram by comparison with the Standard Preparation of Prepared Digitalis.

because of the findings of Watson and James[2]. No leaf samples were collected from any plants since at the time of seed maturity the leaves are in an advanced state of senescence.

Weight of 100 Seeds

The weight of 100 seeds is an accepted diagnostic character[8,9] but its relation with yield or activity of the subsequent plant has not been explored, although some preliminary work was reported by Miller[10]. The present experiment offered such a possibility. The 150 seed samples were allowed to become air dried by spreading in thin layers in the laboratory for some days. Dirty samples were shaken over No. 30 and No. 60 sieves, the former retained portions of capsule wall, placenta, etc., the latter retained the seeds but passed fine dust and, in occasional samples, unfertilised ovules. Seeds on the No. 60 sieve were finally winnowed to free from small portions of capsule wall. There was no evidence of fractionation of seeds by this process of cleaning; clean samples

were not sieved. Preliminary trials suggested that about 1,000 seeds were suitable for counting and weighing. Projection in a photographic enlarger was used to aid the counting; a quarter-plate of perspex or of glass was scratched with a suitable grid of 12 rectangles on the lower side; on the upper surface about 500 seeds were scattered, were picked over with forceps to remove any foreign matter and were mounted in the enlarger. The seed images were thrown on white paper at a suitable magnification and these were readily counted by marking each image. A second slide of about 500 seeds was then counted, the two lots of seeds were mixed and weighed accurately; from these results the weight of 100 seeds was calculated. Replicate results were: sample 38—9·5, 9·4, 9·5, 9·3, 9·2 mg.; sample 54—6·9, 6·8, 6·9, 6·8, 6·8 mg.; sample 55—10·3, 10·1, 10·1, 10·2, 10·1 mg.: it was thus concluded that the method gave dependable results. These values for the 150 samples of seeds are set out in Table II; the two samples of seeds in the lowest weight range did not germinate when subsequently sown and hence the seed of minimal weight which germinates

TABLE II

WEIGHTS OF 100 SEEDS

CLONES 1–150

Weight range mg.	Number of samples	Weight range mg.	Number of samples
3·0–3·9	2	8·0– 8·9	42
4·0–4·9	2	9·0– 9·9	33
5·0–5·9	6	10·0–10·9	17
6·0–6·9	7	11·0–11·9	10
7·0–7·9	31		
		Total	150

Range of weights 3·5 (4·3)–8·6–11·6 mg. per 100 seeds

(4·3) is also shown in the summary of range of weights. The mean weight of 100 seeds collected from the 23 plants rated as A+ was 9·5 mg., for the 19 plants rated as C it was 7·9 mg.; thus suggesting a positive correlation of the robustness of the parent and the weight of its individual seeds.

CULTIVATION, PREPARATION AND ESTIMATION

All clones of seeds were raised in the Museum Experimental Gardens, Mayfield, near Ashbourne, Derbyshire. Sowings were made in pans of John Innes' compost in a heated greenhouse in late February, 1951, germination occurred in 13–20 days and final percentage germinations ranged 28–88–100 per cent. Young seedlings were pricked off as soon as possible into growing-on compost and, when sufficiently matured, were hardened off in a cool greenhouse, cold frame and finally out of doors. Planting out was done May 31–June 1 on to a well prepared bed with the following analytical report "pH 6·61, lime requirement nil, available phosphate 10 p.p.m., available potash 6 p.p.m."; the bed received a dressing of potassium sulphate 1 oz. per sq. yd. shortly before planting out. Ten plants of each strain were placed 18 in. apart in a row; rows were 30 in. apart. The land received normal horticultural tending during the growing period. Harvesting of first year leaf was during

October 9–25, that is approximately 130 days after planting out, and in the afternoon, as far as possible after sunlight. From two plants in each row all leaves, except any decayed outer leaves and the young crown buds, were gathered and weighed to give a measure of total leaf yield per plant as a clone value. Aliquot portions of this material and similar amounts from six further plants in the row were then taken as the clone sample for drying; about 150 g. of dried leaf being produced. The remaining two plants at the end of each row were not harvested. Twenty clone samples were weighed before and after drying to determine the moisture contents, the mean value being used to calculate dry weight per plant for each clone. Drying was carried out in a drying room maintained at 55–65° and with extraction fan; by placing the leaves in shallow, hessian-bottomed

TABLE III

RANGE OF LEAF ACTIVITIES: FIRST AND SECOND YEAR CROPS

CLONES 1–150

First year crop		Second year crop	
Activity range u./g.	Number of clones	Activity range u./g.	Number of clones
18·0–18·9	2	11·0–11·9	3
17·0–17·9	1	10·0–10·9	8
16·0–16·9	5	9·0– 9·9	12
15·0–15·9	21	8·0– 8·9	30
14·0–14·9	38	7·0– 7·9	49
13·0–13·9	36	6·0– 6·9	29
12·0–12·9	25	5·0– 5·9	12
11·0–11·9	16	4·0– 4·9	4
10·0–10·9	3	3·0– 3·9	1
9·0– 9·9	1	No germination	2
No germination	2		
Total	150	Total	150
Activity range: 9·8–13·7–18·9 u./g.		3·1–7·6–11·4 u./g.	

trays and turning the contents night and morning drying was completed in 24–30 hours. Samples were then milled to No. 44 powder, transferred to wide-mouthed bottles and allowed to stand in the drying room for a further 48 hours before putting on the screw caps and transferring to the laboratory for analysis. Moisture contents at 105° of 10 such samples were found to be 3·8–**4·5**–5·3 per cent.

The second year crop of leaf was harvested June 24–July 17, 1952, from the same eight plants (or as many as had survived) in each row; the leaves being gathered from the flowering axes when the lower half of the inflorescence was in full flower but when the upper part of the inflorescence was still in bud. Weight of leaf per plant was determined and drying was done as for the first year crop. The two plants at the end of each row were used for seed; one or more flowering axes of each plant were bagged before the flowers opened, some were left undisturbed and produced little amount of seed, others were carefully opened at intervals and pollination stimulated, after which the bags were replaced. In this way inbred seed for future breeding experiments was collected from every clone in September, 1952.

Objective descriptions of both first and second year plants of each clone were recorded; variation in leaf shape, stem colour shape and hairiness, also differences in inflorescence shape were noted. Rates of development differed between clones but these were least apparent at time of harvest when the slow growing clones had caught up with the others. Leaf weight does, however, show marked differences between clones and is recorded in Table IV for both first and second year plants.

All clone samples were estimated for total glycosidal content by means of the 3, 5-dinitrobenzoic acid process described in Parts I and III of this present series of papers[11,12]. Standard Preparation of Prepared Digitalis was also estimated by the same process at the commencement and at the conclusion of the series of estimations each year and from the results the equivalent activity in u./g. was calculated for each clone. Results for both first year and second year leaves are in Table III.

Discussion

The total glycosidal content shows wide variation amongst the 148 clones about the mean for each year's crop. For first year leaf this is 72–138 per cent; for second year leaf it is 41–150 per cent. This wide range is in general agreement with the findings of other workers[1–5] although a somewhat wider range of activities is here reported from this broad survey of British clones. Activities of first year leaves were satisfactory to good (Table III) and only one sample gave figures of less than 10 u./g., the mean of 148 samples was somewhat higher than the Standard Preparation of Prepared Digitalis and there were several very high-activity clones. A range of these high, medium and low activity clones forms the basis of genetical studies to be reported later.

A comparison of the activities of first year leaf with the geographical origin of the parent seeds is set out in column three of Table I. The mean values for each county are about the same; as the number of samples increases the range of values also increases and thus the larger collections in North Wales include both the poorest (9·8 u./g.) and the two richest (18·9 and 18·6 u./g.) clones. There is no clear evidence that one county produces digitalis of higher activity than another and the evidence is rather of random distribution within natural variation in each county. Comparison of activity of first year leaf and the ecological habitat of the parent also showed no correlation.

Dry weight of leaf yielded per plant (Table IV) was medium to good for first year crop with a range between clones of 48–173 per cent about the mean of 79 g. A comparison of these values with county of origin of parent seed showed a random distribution of values within and between counties with mean values for each county of the same order.

The weight of 100 original seeds (Table II) and either first year leaf yield (Table IV) or leaf potency (Table III); also first year leaf yield and its activity (Tables III and IV) were examined for correlation. These coefficients are given in Table V and it will be seen that no correlation exists of seed weight and leaf yield of progeny; the value 0·131 for seed weight and leaf activity of progeny is probably not significant; and there

is no correlation of leaf yield and its activity. This last is of economic importance since the activity yield per acre is significant and breeding of strains high in both yield and in activity is desirable.

For second year crop the activity range of clones is given in Table III and the dry weight of leaf per plant in Table IV. For both criteria the values are much lower than for first year crop; the mean activity is 7·6 u./g. and only 11 clones have activities of 10 u./g. or over; hence the second year leaves are of inferior quality and are much less to be preferred

TABLE IV

RANGE OF LEAF YIELDS PER PLANT: FIRST AND SECOND YEAR CROPS

CLONES 1–100

First year crop		Second year crop	
Weight of dry leaf, range g.	Number of clones	Weight of dry leaf, range g.	Number of clones
130–139	4	40–49	1
120–129	2	30–39	13
110–119	2	20–29	57
100–109	8	10–19	29
90– 99	9	—	—
80– 89	21	—	—
70– 79	17	—	—
60– 69	21	—	—
50– 59	10	—	—
40– 49	5	—	—
30– 39	1	—	—
Total	100	Total	100
Dry weight range: 38–79–137 g.		12–23–47 g.	

than those of the first year crop. Such a finding is in agreement with those of some other workers[13–16] but conflicts with other publications. Since leaf yield is also low with a variation of 52–204 per cent about the mean of 23 g. per plant, the second year crop is of much less economic significance than is that of the first year.

There was no obvious correlation of leaf activities and objective descriptions of either first year or second year plants and it is not possible to forecast from plant appearance the amount of glycosides present in the

TABLE V

CORRELATION COEFFICIENTS

CLONES 1–100

Correlation	r
Seed weight: Leaf yield, first year	−0·034
Seed weight: Leaf activity, first year	0·131
Leaf yield, first year: Leaf activity, first year	0·045
Leaf activity, first year: Leaf activity, second year	0·335

leaves. Despite the low potencies of second year leaves there is a correlation of them and the activity of the first year crop from the same clone. This is shown in Table V and it suggests a measure of genetical control of glycosidal content in addition to seasonal and environmental influences.

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STUDIES IN THE GENUS DIGITALIS

PART VII. VARIATIONS IN GLYCOSIDAL CONTENT WITHIN CLONES OF *Digitalis purpurea*

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First year leaves of *Digitalis purpurea*, collected in July or August, contain smaller amounts of glycosides than those collected in September, October or early November when the values are relatively constant, with a coefficient of variation of about 7 per cent. Second year leaves contain uniform amounts of glycosides until the plant is in full flower, coefficient of variation about 8 per cent, after which glycosidal content decreases. Weight of leaf per plant increases throughout the first year of growth: it is less for second year plants and reaches a maximum in the early stages of flowering. Defloration of second year plants changes the accumulation pattern of glycosides to that of first year plants. Coefficient of variation in glycosidal content within groups of up to 10 plants in one clone was 9·4 per cent for first year plants (265 plants from 13 clones, grown on four sites, three growing seasons) and was 13·3 per cent for second year plants. Between groups of 10 plants coefficients of variation were 8·5 per cent (first year) and 11·5 per cent (second year). Season of growth and site where grown both influence the glycosidal content of plants; the latter is probably due to climate rather than to nutrition. In comparative experiments on different sites the influence of the site may be eliminated by expressing all results as percentages of the corresponding mean site values; this has been done for 45 comparisons on each of four sites when the coefficients of variation were first year plants 10·5 per cent, second year plants 12·0 per cent. It is concluded that these figures are a measure of the coefficient of variation of total glycosidal content in a clone of *Digitalis purpurea* leaves.

THE total glycosidal contents of 150 different clones of *Digitalis purpurea* leaves were reported in Part VI of this series[1] and some of these clones have been taken for further selection and breeding experiments. To assess the validity or otherwise of any such breeding experiments it is necessary to study in detail the natural variation which may occur within single clones.

Glycosidal content may be influenced by external factors such as nutrition, environment and climate, all of which are closely interrelated and which we must seek to control when examining the possible inheritance of a factor for high or low glycosidal content. Wasicky[2] considered that the method of cultivation influenced the proportions of the different glycosides present in the glycosidal mixture and he suggested that plants should be cultivated under standard conditions. In Part IV of this series[3] we have studied the influence of fertilisers upon leaf yield and upon glycosidal content of those leaves and the remarkable constancy of glycosidal content under all treatments excepting that with lime was found to be most significant. Thus no further study of nutritional factors seems desirable but the complex interaction of climate and nutrition to be found at different sites of cultivation needs to be investigated. Accordingly

much of the following work was carried out at four different sites in England. Also the experiments were repeated for several years to determine the influence of season upon glycosidal content. Many of the determinations have been made upon both first and second year plants.

The amount of glycosides present in leaves from a plant at different ages has not been fully investigated. Tattje[4] found a maximum glycosidal content in first year plants in July, 140–150 days after sowing; this was followed by a decrease and a subsequent second but low maximum in October. He also found that first year leaves were richer in total glycoside than were second year leaves[5]. Yamamoto and others[6] found a maximum in first year leaves in the month of August, followed by a gradual decrease and then an increase in second year leaves up to flowering in May. Klepsaite[7] found a maximum in September and a decline in October in first year leaves, whilst for second year leaves the glycosidal content was greater before blooming than afterwards. In studies of *Digitalis lanata* Court and Allemann[8] found that in second year leaves the glycosidal content fell rapidly as flower stems developed. Since all previous work in this series of papers has been based upon leaves of first year plants collected in late September, and of second year plants harvested when the upper flowers were opening, it was decided to make a detailed study of the total glycosidal content of leaves gathered throughout the life history of this biennial plant.

Apart from the age of a plant and also the possible inheritance of glycosidal content, to be investigated subsequently, the possibility of natural variation of glycosidal content within a clone must be envisaged and due to internal factors of which we have no knowledge. No previous work upon plant by plant variation of glycosidal content could be found and thus a large scale experiment was set up to investigate this fundamental fact within a number of clones of *D. purpurea.*

Experimental

The seed clones used were those reported in Part VI[1]. Horticultural details were the same as those set out in that paper and in Part IV[3] and all young plants were reared at site B at Mayfield near Ashbourne, Derbyshire. For the work carried out on other sites the young plants were chosen at random from this nursery and were rapidly transferred to the planting out sites. These were site M, the Farm Institute, Morley, Derbyshire; site S, Department of Horticulture, University of Nottingham, Sutton Bonington; site W, the gardens of Allen and Hanbury, Ltd., Ware. Harvesting and rapid drying were carried out as described previously[1,3]. Total glycosides were estimated by the 3,5-dinitrobenzoic acid process described previously[8] and results were expressed as "units" per gram, by comparison with the Standard Preparation of Prepared Digitalis.

Variations in Leaf Yield and Activity with Age of Plant

Leaves were collected twice a month from the time of planting out (July) until the first year plants died back because of frosts in November.

Collections were recommenced the following spring from rosette leaves and then from axial leaves as the inflorescence developed; samples were taken until the plants were completely senescent. The state of maturity of the inflorescence was noted and is recorded in the second part of Table I. The experiment was made at site B and was repeated in three successive

TABLE I

VARIATIONS IN LEAF YIELD AND ACTIVITY WITH AGE OF PLANT
(BI-MONTHLY HARVESTS)

Season	1951–52		1952–53		1953–54	
Clone	A0004		B0033		A1033	
Planted out	9.6.1951		10.6.1952		1.7.1953	
Date of harvest	Dry weight of leaf per plant g.	Activity u./g.	Dry weight of leaf per plant g.	Activity u./g.	Activity u./g.	Notes
First Year Crop						
July 1	1·6	10·4	—	—	7·7	Activity values in years 1951–52 and 1952–53 are based upon harvests of 4 plants on each occasion. In 1953–54 a group of 34 plants was reared and each assay is based upon a sample of 68 leaves, 2 leaves being taken from each plant.
July 16	3·8	9·7	3·5	9·1	—	
August 1	10·8	11·9	—	—	6·8	
August 16	31·0	10·5	7·5	10·5	9·6	
September 1	58·0	12·3	16·3	14·8	9·4	
September 16	46·5	11·1	25·5	12·9	8·3	
October 1	66·5	12·1	18·5	14·2	—	
October 16	66·0	13·2	27·5	13·8	8·9	
November 1	82·5	14·4	54·8	13·8	—	
November 16	84·0	12·7	51·0	14·7	—	
Second year crop						
March 16	18·0	9·4	—	—	5·0	
April 1	9·5	10·1	15·0	8·5	—	
April 16	12·0	8·8	15·0	7·9	—	
May 1	13·0	8·2	20·0	7·3	—	
May 16	25·0	8·3	30·5	8·9	6·1	Inflorescence axis showing
June 1	40·0	7·6	57·0	7·6	7·3	Inflorescence developing
June 16	27·0	8·5	—	—	6·8	Upper flowers opening
July 1	31·0	8·8	47·0	9·2	—	In full flower
July 16	14·0	7·7	40·0	7·9	6·6	In full flower
August 1	—	—	30·0	6·4	6·9	Some fruit well developed
August 16	10·0	7·7	—	—	—	Some fruit mature
September 1	9·0	6·5	—	—	—	Fruit mature, plant senescent

The "units" were estimated by the 3,5-dinitrobenzoic acid process and expressed as units per gram by comparison with the Standard Preparation of Prepared Digitalis.

pairs of growing seasons 1951–52, 1952–53 and 1953–54. In the two first experiments all the leaves, apart from dead outer ones and young buds, from a block of four plants were taken for each bi-monthly harvest and hence the weight of dry leaf per plant at that date was also recorded (columns 2 and 4 of Table I). The plants were then left until the following year and were again harvested in the same succession as in the first year. This design of experiment does not take account of any variation within the garden upon which some 16 blocks of four plants were raised. Thus, for the experiment in 1953–54 a block of 34 plants was reared and at each bi-monthly harvest two typical leaves were taken from each plant. Under these conditions the yield of dry leaf per plant at each harvest could not be determined, but the total variation between plants was represented in each sample.

The results are set out in Table I from which it is seen that the weight of leaf yielded by a first year plant increases steadily with the age of the plant up to the month of November. The glycosidal content of first year leaves is lowest for young plants (July and August) but retains a relatively constant, high value for plants harvested during the months of September, October and the first half of November. The three sets of results show some variations in values during this September to November period, but the variations are not constant and the results are evidence of one uniform high value rather than of a pair of peak values during this period. It is thus satisfactory to harvest first year plants at any time during the period September to mid-November and the value for glycosidal content will then have a coefficient of variation, v, of about 6·8 per cent. (Table I, first year crop, September to November values; column 3 mean = 12·6, s = 1·1, $v = 1{\cdot}1 \times 100/12{\cdot}6 = 8{\cdot}7$ per cent; similarly column 5 $v = 5{\cdot}0$, column 6 $v = 6{\cdot}7$.) For second year plants the weight of leaf per plant increases with age until the time of flowering (June) after which it decreases markedly. The maximum weight yield of leaf does not exceed that of the first year plants, and generally it is less. The amount of glycosides present in these leaves remains relatively constant until the time of full flowering after which there is a decline. Thus it is satisfactory to harvest second year leaves during the period of inflorescence development (approximately mid-May to mid-July) and the value for glycosidal content will have a coefficient of variation of about 7·7 per cent.

Defloration

If young flowering axes are removed as they arise from second year plants, the basal rosette of leaves continues to develop throughout the growing season and such plants may live for several years by repetition of this treatment[9]. As a parallel to the bi-monthly harvests reported above some groups of second year plants of clone A0004 were freed from young inflorescence axes as they developed in 1952 and leaf samples were taken at monthly intervals. The same groups of plants were similarly treated in 1953 when they were in their third year of growth; the defloration of second year plants of clone A1033 was also carried out in 1953. Assays are given in Table II and it will be seen that the pattern of glycosidal

content at monthly intervals resembles that of first year rather than of second year plants; thus there is no falling off in values after July and in two of the series there is an increase in values in August or September. It may thus be concluded that the internal factor of plant maturity in terms of flowering and fruiting exerts a marked influence on the total glycosidal content of its leaves.

Variations in Leaf Activity within a Clone

Individual plants. Ten individual plants were reared in a row from each clonal batch of seed, leaf samples were collected from first year plants in late September or from second year plants during the period of flowering. Each individual sample was estimated for total glycosides. The investigation was made on site B during 1953, 1954 and 1956: in 1955 the investigation was made on the four sites B, M, S and W and in 1956 these plants

TABLE II

VARIATIONS IN LEAF ACTIVITY WITH AGE OF DEFLORATED PLANTS

(c.f. Table I)

u./g.

Season	1952	1953	1953
Clone	A0004		A1033
Crop	Second year	Third year	Second year
April 16	—	—	8·4
May 16	—	—	8·9
June 1	7·6	9·5	—
July 1	—	8·5	8·0
August 1	8·5	10·6	8·1
September 1	10·6	—	8·2

on sites B and M were continued as a second year crop. Individual figures for estimations of the 10 plants within each row were recorded and their coefficients of variation, *v*, were calculated. Typical results for five clones are shown in Table III, Part A. Totals of 265 first year plants from 13 clones and 124 second year plants from 10 clones were estimated and Table IV expresses in summarised form the means and standard deviations for each group of plants; where the number of plants in each group differs from 10, the actual figure is shown in brackets.

It will be noted that for first year plants on site B in three years the values of *s* vary between 0·58 and 1·34 and on four sites in one year *s* varies between 0·52 and 1·88. The calculated coefficients of variation of activity are for site B (three years) 4·5–**8·8**–11·9, and for four sites in one year 5·3–13·7. The mean value for all sites in the four years is **9·4** per cent. The values of *s* for first year crop in 1955 on four sites (Table IV, part B) suggest that the clones A2042 and A2060 are less variable than the other three clones: the coefficients of variation on the four sites are: clone A2007 11·0 per cent, A2012 11·3 per cent, A2034 10·3 per cent, A2042 9·0 per cent and A2060 7·2 per cent.

For second year plants on site B the values of s vary between 0·40 and 1·86 and for 1956 crop on the two sites the range of s is 0·40 to 1·45. The coefficients of variation of mean activity values on site B for second year plants grown in three seasons are 4·5–**14·3**–27·8. The mean for all second year plants on both sites is **13·3** per cent: thus the glycosidal content of second year plants shows a greater variation than for first year plants. Results for second year plants also confirm the conclusion drawn from first year assays that there is less variability within clones A2042 and A2060 than within the other three clones grown in seasons 1955–56.

TABLE III

VARIATIONS IN LEAF ACTIVITY: FIRST YEAR CROP, 1955

SITE B

u./g.

A. Individual plants in a row (c.f. Table IV)

Clone	Plant number										Mean	Standard deviation s
	1	2	3	4	5	6	7	8	9	10		
A2007 ..	10·0	9·1	11·6	12·4	12·6	11·4	13·3	11·4	13·0	12·6	11·7	1·34
A2012 ..	11·9	11·9	11·6	13·6	14·9	13·5	12·3	11·3	11·7	13·9	12·7	1·22
A2034 ..	11·0	12·0	9·6	12·1	13·4	12·9	12·9	12·6	14·0	13·7	12·4	1·33
A2042 ..	11·4	10·0	9·2	10·3	10·0	9·7	9·2	11·0	9·3	11·4	10·2	0·86
A2060 ..	10·3	10·1	9·3	9·6	10·0	10·3	10·4	9·2	8·9	8·0	9·6	0·77

B. Individual rows each of 10 plants (c.f. Table V)

Clone	Row Number					Mean	Standard deviation s
	1	2	3	4	5		
A2007	11·7	13·7	13·6	13·4	13·4	13·2	0·83
A2012	12·7	13·6	13·9	14·6	12·4	13·4	0·90
A2034	12·4	13·9	14·6	13·4	13·3	13·5	0·81
A2042	10·2	12·4	12·4	11·9	11·7	11·7	0·90
A2060	9·6	10·1	9·4	10·7	10·1	10·9	0·51

Groups of plants. It has been the normal custom in this series of papers to make estimations of glycosides on a leaf sample drawn representatively from a group of plants of the clone, the number in such a group being up to 10. Such values should have a lower standard deviation than assays based on individual plants. The individual plant experiments, reported above, were also designed to examine this variation between groups of plants within the clone. For each clone five rows each of 10 plants were reared. The individual plants in one row were harvested for the investigation reported above (and the mean values used in this work); representative handfuls of leaves from each of the 10 plants in row 2 were mixed and dried to form the sample for that row and samples for rows 3, 4 and 5 were similarly drawn. Plants from five clones of seeds were investigated in this way upon the four sites during 1955, and on site

TABLE IV

VARIATIONS IN LEAF ACTIVITY: INDIVIDUAL PLANTS (10 PER ROW)
MEAN VALUES AND STANDARD DEVIATIONS WITHIN ROWS

(c.f. Table IIIA)

A. SITE B

First year crop				Second year crop			
Year	Clone	Activity u./g.	*s*	Year	Clone	Activity u./g.	*s*
1953	A1005	13·6 (5)	1·29	1953	B0011	9·5	1·30
	A1102	13·0	1·22		B0047	7·9	1·59
1956	B2005	13·0	0·58	1954	A1012	5·5	0·75
	B2012	11·8	1·22		A1109	6·8	1·10
	B2035	12·4	1·03		A1113	6·7	1·86
	B2060	10·1	0·62				
	B2100	10·2	0·60				
	B2150	8·1	0·96				

B. FOUR SITES. 1955–56 CROPS

Clone	A2007		A2012		A2034		A2042		A2060	
Site	Activity	*s*	Activity	*s*	Activity	*s*	Activity	*s*	Activity	*s*
First year crop, 1955										
B ..	11·7	1·34	12·7	1·22	12·4	1·33	10·2	0·86	9·6	0·77
M ..	9·7	1·09	12·4	1·31	14·6	1·05	11·5	0·75	10·4	0·85
S ..	12·4	1·21	13·1	1·45	15·7	1·70	11·1	0·99	9·8	0·52
W ..	16·2	1·84	13·1	1·79	15·3	1·88	13·2	1·57	—	—
Second year crop, 1956										
B ..	8·4	1·45	10·0 (6)	0·82	10·8 (9)	1·32	6·7 (7)	0·53	6·6 (7)	0·40
M ..	5·8 (2)	1·27	6·8 (6)	0·77	7·8 (8)	0·53	7·1 (9)	0·65	7·5	0·51

Note: Figures in brackets are numbers of plants estimated where other than 10.

B the 25 rows were continued to the second year crop in 1956. Representative results for rows, the mean value and its standard deviation for each clone are set out in Table III, part B. A summary of these mean values and of their standard deviations is given in Table V.

For first year plants on the four sites values of *s* about the mean values per row within a clone vary between 0·23 and 2·25. Coefficients of variation of activity are 2·3–**8·5**–17·3 for first year plants; the corresponding values for second year plants are 8·0–**11·5**–15·6 per cent; it is thus confirmed that the mean standard deviation, *s*, and the coefficient of variation, *v*, for groups of 10 plants are less than for individual plants in both first and second year crops. When values of *s* for individual plants are compared with those for rows in the 19 instances of the 1955 first

year crop, the former is greater than the latter in 13 cases; whilst 15 values of v are greater for individuals than for rows. For the four aberrant instances, three of which were on site M, it must be supposed that the external factors influencing glycosidal content were not uniform between rows: it should also be noted that the actual activity values on site M were very low in 1955 and it is probable that such seasonal edaphic factors producing these low values may also accentuate the natural variation within the clones. The low variability found within clones A2042 and A2060 in Table IV was not found in Table V.

TABLE V

VARIATIONS IN LEAF ACTIVITY: INDIVIDUAL ROWS (EACH OF 10 PLANTS) MEAN VALUES AND STANDARD DEVIATIONS BETWEEN FIVE ROWS

(c.f. Table IIIB)

FOUR SITES. 1955–56 CROPS

u./g.

Clone	A2007		A2012		A2034		A2042		A2060	
Site	Activity	s	Activity	s	Activity	s	Activity	s	Activity	s
First year crop, 1955										
B ..	13·2	0·83	13·4	0·90	13·5	0·81	11·7	0·90	10·9	0·51
M ..	9·3	0·35	10·3	1·49	12·3	1·55	11·1	0·64	8·1	1·40
S ..	11·9	0·82	11·8	1·17	13·8	1·23	11·3	1·85	10·0	0·23
W ..	16·1	0·97	16·3	2·25	16·7	1·05	13·3	0·79	—	—
Second year crop, 1956										
B ..	7·5	0·60	8·8	0·81	9·3	1·40	8·0	1·25	7·7	0·75

Variations in Leaf Activity with Site of Cultivation and Season of Growth

To investigate the influences of season and of site of cultivation upon the glycosidal content of individual clones of *D. purpurea*, seeds from 15 clones representative of high, medium and low glycosidal content were employed. Rows of 10 plants of each clone were grown on each of the four sites in 1952 and clone samples were collected for assay in late September. Plants were continued into second year in 1953 and representative clone samples taken when the plants were in flower. The investigations were repeated in 1953–54 and again in 1954–55. Results are given in Table VI; a number of second year plants failed to mature on one or more of the sites and such clones have been omitted from the lower half of the Table.

It must be stressed that Table VI does not attempt to illustrate the inheritance within a clone of a factor for glycosidal content upon each of the four sites of cultivation. All seed samples were taken from inbred clones growing at site B for inheritance studies and hence first year plants grown on sites M, S or W in 1953 or 1954 do not stem from those grown

TABLE VI

VARIATIONS IN LEAF ACTIVITY: FOUR SITES; THREE SEASONS
CLONE SAMPLES FROM 10 PLANTS

u./g.

First year crop

Site ..	1952 B	1952 M	1952 S	1952 W	Site ..	1953 B	1953 M	1953 S	1953 W	Site ..	1954 B	1954 M	1954 S	1954 W
Clone ..					Clone					Clone				
B0002	13·3	12·7	12·0	10·7	A1002	11·7	9·2	14·2	10·7	B1002	8·6	8·2	9·9	11·3
B0005	14·3	13·6	15·2	13·9	A1005	12·4	9·5	10·8	13·8	B1005	9·6	13·3	13·6	12·3
B0007	13·5	13·4	12·4	13·6	A1007	9·8	8·1	12·5	13·1	B1007	10·4	9·5	12·7	13·7
B0012	10·4	11·9	10·8	12·3	A1012	9·6	8·0	15·8	8·9	B1012	9·3	10·3	10·1	12·8
B0026	10·3	10·9	15·0	14·8	A1026	10·7	—	14·6	10·5	B1026	10·1	10·8	13·3	13·6
B0042	11·0	10·1	14·5	11·7	A1042	8·3	6·3	11·5	9·1	B1042	11·4	12·3	—	13·3
B0046	10·6	11·0	10·4	11·3	A1046	8·3	5·9	12·4	11·3	B1046	11·7	11·4	10·3	—
B0060	9·1	11·6	12·1	10·7	A1060	8·2	7·6	13·4	10·4	B1060	9·9	12·9	11·2	10·3
B0062	10·2	14·1	14·4	12·3	A1062	11·0	7·5	14·7	13·5	B1062	12·1	9·8	12·2	12·6
B0075	12·3	13·1	12·5	14·5	A1075	8·8	6·2	15·2	10·5	B1075	11·2	11·1	11·6	14·2
B0093	11·1	11·8	11·5	13·2	A1093	12·1	8·5	16·0	13·2	B1093	11·5	12·4	12·0	14·8
B0094	11·3	10·4	12·5	12·5	A1094	10·7	10·7	14·4	9·9	B1094	11·2	12·7	11·2	11·9
B0100	11·1	10·5	9·5	7·9	A1100	7·8	6·6	8·1	5·5	B1100	9·7	9·1	9·7	11·5
B0102	13·2	10·1	14·1	—	A1102	13·1	9·5	15·0	10·9	B1102	13·8	12·5	11·8	15·2
B0150	—	8·9	9·1	11·0	A1150	7·9	8·5	10·2	11·7	B1150	10·1	9·7	9·7	14·0
Mean	11·6	11·6	12·4	12·2		10·0	8·0	13·3	10·9		10·7	11·1	11·4	13·0

Second year crop

	1953					1954					1955			
B0002	10·4	9·5	14·7	9·2	A1002	7·4	6·8	10·6	—	B1002	9·4	9·1	9·1	11·0
B0005	11·1	10·7	13·5	10·2	A1005	8·7	6·0	8·3	—	B1005	9·0	11·1	10·4	14·1
B0042	9·7	7·3	9·5	9·8	A1042	7·1	4·6	7·0	—	B1042	8·6	8·6	7·9	13·1
B0046	7·4	9·1	9·6	8·9	A1046	6·8	5·5	9·0	—	B1046	6·9	6·0	8·4	—
B0062	9·9	11·8	15·5	9·9	A1062	9·5	8·0	12·6	—	B1062	10·7	11·7	10·4	13·4
B0093	11·1	11·5	8·9	9·5	A1093	8·7	6·4	8·2	—	B1093	7·6	8·0	7·1	11·7
B0094	9·9	7·3	10·3	9·6	A1094	9·3	5·4	7·8	—	B1094	7·1	8·0	7·4	14·0
B0100	8·1	8·2	8·9	8·7	A1100	5·5	5·2	7·6	—	B1100	6·4	5·7	7·4	11·1
B0102	12·9	9·0	11·5	—	A1102	7·5	4·5	9·3	—	B1102	10·3	9·4	10·3	17·0
B0150	—	9·6	8·1	8·2	A1150	5·1	7·1	7·7	—	B1150	8·1	9·0	7·0	13·4
Mean	10·1	9·4	11·1	9·3		7·6	6·0	8·8			8·4	8·7	8·5	13·2

Mean site values: B = 9·9, M = 9·4, S = 11·2, W = 11·8

Yearly Means: First year crop: 1952 = 12·0, 1953 = 10·6, 1954 = 11·5
Second year crop: 1953 = 10·0, 1954 = 7·5, 1955 = 9·6

on the same sites in 1952. In consequence a strict comparison may only be looked for within a clone on four sites in the same year: although the last three figures of the clone reference numbers are those of the original seed collections reported in Part VI of this series[1] and hence there is a relationship in reading completely across this Table.

A consideration of the results in Table VI reveals that there is a clear influence of the site upon the glycosidal content of plants grown thereon, and the sites may be ranked for glycoside production by comparing the mean site values. Thus by using first year plant values in each of the three years, sites B and M are variously ranked 4th or 3rd and sites S and W 2nd or 1st, whilst second year plants give rather wider variations in ranking in the three years. Mean site values for both first and second year plants in the four years of the experiment are set out in Table VI and these show a descending ranking of the sites in the order W, S, B and M, the difference between the first two and the second two being marked. Since all plots consisted of adequately manured and well tilled garden plots it seems possible that the variations were due to external factors other than nutrition and in this context it should be noted that site S is further south than either B or M; whilst site W is still further south.

Table VI also indicates a marked influence of the season of growth upon the glycosidal content of the plants. Ignoring differences between clones and between sites it is seen that for first year crop the glycoside production is in the descending series 1952, 1954, 1953; the year 1953 producing low potencies. A similar ranking based upon second year crop gives 1953, 1955, 1954 with 1953 as the best and 1954 as the worst year. This apparent contradiction between assessments based on the first or second year crops is explicable on the basis that the season exerts an influence upon first year plants and this is carried on into the subsequent year of growth. Thus 1952 was a "good" year as evidenced by yearly means for first year crops, and those plants retained this "good" influence in their second year of growth in 1953 although this year was a "bad" year as indicated by first year crop results. Similarly this 1953 "bad" influence on first year plants persisted in them in 1954 as second year crop despite the fact that 1954 was a "fairly good" year as shown by the first year crop.

We may thus summarise the conclusions of this section in stating that plants of *D. purpurea*, grown from the same batch of seeds, may differ in glycosidal content if grown upon different sites in the same year; they may also differ in glycosidal content if grown upon the same site but in different years. These two external factors must be considered in assessing the internal factors of the clone for glycoside production.

Discussion

The experimental results reported above have been discussed in each of the sub-sections, since the conclusions arrived at in one investigation determined the approach to the succeeding one. Each may now be considered as part of the whole work.

Bi-monthly harvests (Table I) have shown that first year plants have reached a uniform level of glycoside content by the commencement of

September if grown under normal conditions of cultivation. Since the weight of leaf per plant increases continually throughout the growing season up to mid-November it follows that each plant produces an increasing amount of glycoside throughout this period. We do not know if this "amount" constitutes a food store laid down by the plant, or if the "percentage" constitutes a threshold value in some stage of the metabolic cycle of the plant. It is interesting to note the different mobilities of the primary and secondary digitalis glycosides and aglycones of the A and B series and to speculate upon the influence of these on translocation and storage patterns in the living plant, but much more evidence is required upon the subject. We do, however, conclude that leaves collected from September onwards contain a normal percentage of glycosides; whereas commercial harvesting should take place as late as possible when the crop weight is at its maximum. For second year plants the percentage of glycosides present in the leaves remains fairly constant until the time of full flowering, after which the content decreases. Since the weight of leaf per plant increases up to the time of early flowering it follows that in this period there is an increased amount of glycoside produced by each plant; whilst during the later stages of senescence the glycosides disappear. Hence, such second year plants should be harvested when in early flowering; although commercially the crop yield and glycosidal content are both lower than for first year plants. When second year plants are prevented from flowering by removal of flowering axes as they appear, such plants behave as first year plants in growth habit and in accumulation pattern of glycosides. This is of interest in that the internal factor leading to senescence of the plant and decrease in glycosidal content has been influenced by external treatment.

The external factor, or group of factors, covered by the word season, influence the percentage of glycosides produced in the same clone of plants and this is especially significant in the first year of plant growth (Table VI). It follows that inheritance studies which spread over a number of growing seasons must take note of this fact. It is probable that light, warmth and rainfall are all concerned in the influence of season but the meteorological records have not been analysed for the five years and four sites involved in this work.

Although previous work showed that glycosidal content could not be much influenced by either manurial treatments or by starvation[3], the site of cultivation influences this content (Table VI) and it has been suggested that this is an influence of climate rather than of nutrition. Its existence means that individual figures for amount of glycoside present are not directly comparable between different sites in any one year.

When the external factors of site and season are excluded from our considerations and when plants are harvested at the time of constant and maximum glycosidal content, there still remains to be measured the individual variation within a clone. Table IV records the standard deviations within groups of 10 plants of one clone and, although the experiment extended over four different sites and over four growing seasons with corresponding differences in mean values expressed in terms

of u./g. in each instance, the different values of s within each clone were about the same. To obviate these site : season variables the values of s were converted into coefficients of variation ($v = 100\ s/\bar{x}$) and these values of v were of still closer similarity within clones and also between clones. The mean value of v for 13 clones of first year plants grown on

TABLE VII

VARIATIONS IN LEAF ACTIVITY WITHIN A CLONE
MEAN ACTIVITIES (PER CENT), STANDARD DEVIATIONS AND COEFFICIENTS OF VARIATION

(c.f. Table VI)

First year crop

Clone	Mean	s	v	Clone	Mean	s	v	Clone	Mean	s	v
	1952				1953				1954		
B0002	102·2	12·2	11·9	A1002	109·3	8·6	7·9	B1002	82·0	6·2	7·6
B0005	119·0	4·5	3·8	A1005	112·7	21·2	18·8	B1005	105·9	16·0	15·1
B0007	135·9	7·5	5·5	A1007	103·4	11·6	11·2	B1007	99·9	11·2	11·2
B0012	95·0	7·8	8·2	A1012	99·1	15·3	15·4	B1012	91·7	5·2	5·7
B0026	106·3	17·3	16·3	A1026	104·4	7·1	6·8	B1026	103·3	9·9	9·6
B0042	98·7	12·8	13·0	A1042	83·0	3·2	3·9	B1042	106·5	4·3	4·0
B0046	90·7	4·7	5·2	A1046	88·4	12·9	14·6	B1046	100·8	9·6	9·5
B0060	90·9	9·9	10·9	A1060	93·3	8·0	8·6	B1060	96·5	15·3	15·9
B0062	106·6	15·3	14·4	A1062	109·6	12·3	11·2	B1062	101·3	11·0	10·9
B0075	109·7	7·9	7·2	A1075	94·0	15·6	16·6	B1075	103·9	4·0	3·8
B0093	99·6	6·9	6·9	A1093	117·2	7·3	6·2	B1093	109·6	5·1	4·7
B0094	97·6	5·7	5·8	A1094	110·0	17·8	16·2	B1094	102·2	9·8	9·6
B0100	81·9	14·0	17·1	A1100	68·0	14·9	21·9	B1100	86·6	3·8	4·4
B0102	104·9	15·4	14·7	A1102	115·7	12·9	11·2	B1102	115·5	10·6	9·2
B0150	79·6	9·3	11·7	A1150	92·3	16·7	18·1	B1150	93·7	10·2	10·9

Second year crop

	1953				1954				1955		
B0002	108·9	15·8	14·5	A1002	110·4	11·8	10·7	B1002	101·7	12·7	12·5
B0005	113·8	5·6	4·9	A1005	102·9	10·4	10·1	B1005	116·0	10·6	9·1
B0042	91·2	12·1	13·3	A1042	83·2	8·9	10·7	B1042	98·4	4·0	4·1
B0046	88·1	10·9	12·4	A1046	94·5	6·8	7·2	B1046	83·3	14·9	17·9
B0062	117·4	18·7	15·9	A1062	133·8	9·1	6·8	B1062	121·5	14·2	11·7
B0093	103·7	17·7	17·1	A1093	104·8	10·8	10·3	B1093	88·7	3·7	4·2
B0094	92·9	11·0	11·8	A1094	100·3	19·1	19·0	B1094	92·4	9·7	10·5
B0100	85·3	6·4	7·5	A1100	81·8	8·2	10·0	B1100	78·2	9·6	12·3
B0102	109·0	16·7	15·3	A1102	93·1	16·1	17·3	B1102	120·2	8·7	7·2
B0150	87·8	14·6	16·6	A1150	91·0	25·8	28·4	B1150	95·9	9·5	9·9

one to four sites in four years was 9·4 per cent. When groups of 10 plants were taken and five such groups examined the value of v was 8·5 per cent, based upon groups of four plants in two years and upon a group of 34 plants in the third year. For second year plants the corresponding values of v are: individual plants 13·3 per cent (from Table IV), groups of 10 plants 11·5 per cent (from Table V), groups of four or more plants 7·7 per cent (from Table I).

This individual variation within a clone may be abstracted by a further consideration of Table VI. The total mean site values for each of the four sites have been used to show the influence of site upon percentage of glycosides present in plants growing thereon; and this influence we believe to be climatic rather than nutritional. Proportional ranking of sites by their annual mean values differs from year to year even when only first year plants are considered. It seems reasonable, however, to expect that all plants of the same year are influenced in a similar manner by the site upon which they are growing and this is supported by the results of Tables IV and V. Such site variations can then be eliminated by converting all clone values into a percentage of the mean site value for that year and crop of plants. Table VI was recalculated on this basis and within each annual group each clonal percentage mean was calculated together with its standard deviation and hence the coefficient of variation of that clone. These are set out in Table VII. For the 45 first year clones the values of coefficient of variation, v, = 3·8–**10·5**–21·9 per cent; the figures for individual years are: 1952, 3·8–**10·2**–17·1; 1953, 3·9–**12·6**–21·9; 1954, 3·8–**8·8**–15·9; hence 1953, which was a poor growing season, showed a higher annual mean value of v and also the highest individual value compared with the other two years. For the 30 second year clones the values of v are: 1953, 4·9–**12·9**–17·1; 1954, 6·8–**13·1**–28·4; 1955, 4·1–**9·9**–17·9; three years, 4·1–**12·0**–28·4 per cent. The greater variability noted in the 1953 first year crop is continued in the 1954 second year crop. This mean value of v = 10·5 per cent for first year plants, based upon 45 groups of 10 plants each grown on four sites and corrected for site influence, is in good agreement with the value 8·5 per cent for groups of 10 plants grown in five rows on each of four sites the results being calculated within sites only (discussion on Table V). The higher value for these present calculations may be due only to the much larger group of 45 comparisons or there may still be some residual influence of the four sites causing a somewhat higher coefficient of variation of results within each clone. Similarly there is good agreement between values of v for second year crop; these are 12·0 per cent in this experiment and 11·5 per cent between rows (discussion on Table V). There is no marked correlation in values of v for related clonal groups of plants grown in different years, nor is the relationship between corresponding first and second year plants very pronounced, but the two clones 100 and 150, which are poorest in yield of glycosides in each season, also tend to have high values of v. It is reasonable to conclude that the normal coefficient of variation of glycosidal content to be expected within a clone of digitalis plants is about 10 per cent for first year crop and 12 per cent for second year crop.

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After Dr. Rowson presented the papers there was a DISCUSSION. The following points were made.

The activity of the first year leaves was twice that of the second year plants and the leaf yield was three times as great. The leaves should be harvested as late as possible in the year before the frosts. The weight of leaves from the deflorated plants in the second year was about the same as the yield from the first-year plants.

THE STRENGTH OF COMPRESSED TABLETS

PART I. THE MEASUREMENT OF TABLET STRENGTH AND ITS RELATION TO COMPRESSION FORCES

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A motorised single-punch eccentric tablet machine has been fitted with instruments to measure the forces operating during the compression cycle. Apparatus has also been designed to measure the resistance to crushing of the final compact. Sodium chloride was compressed at varying pressures and the relationship between compaction forces, ejection force, strength and voidage investigated.

FUNDAMENTAL research on the problems of tabletting was begun in 1950 by Higuchi, Arnold, Tucker and Busse[1] and in subsequent investigations many aspects of formulation and production were described. Train[2] has studied the transmission of forces through a powder under compression and other contributions have been made by Munzel and Kagi[3], and by Seth[4].

This investigation is concerned with the factors affecting tablet strength. Sodium chloride was used since it forms a satisfactory tablet without the addition of excipients, and in this way, a simple system could be studied.

The Instrumentation of a Tablet Machine

The instrumentation of a tablet machine has been described by Higuchi, Nelson and Busse[5]. Strain gauges were bonded to the frame of a single-punch eccentric machine to give a measure of the applied force. The disadvantage of this was the non-linear relation between frame distortion and the applied force. We used a Lehman single-punch eccentric tabletting machine, driven at a constant speed through a Kopp Variator. A $\frac{1}{2}$ inch plane-faced, cylindrical punch and die set was chosen and two strain gauges, connected in series (Tinsley, Type 6H, gauge factor 2·16, resistance 50 ohms), were bonded to the upper punch. The compensating resistance consisted of two similar gauges on a piece of punch steel of the same dimensions. The active (upper punch) and compensating resistances formed two arms of a Wheatstone Bridge connected to one channel of a carrier amplifier (New Electronic Products, type 1070). The bridge was activated by a 3,000 c.p.s. source of 4 volts (r.m.s.). The magnitude and phase of the bridge output depended on the resistance of the strain gauges and after amplification and phase sensitive rectification, the signal was applied to a recording galvanometer having a natural frequency of 250 c.p.s. The optical arm was 20 cm. in length and had an ultra-violet light source. The deflection was recorded on a sensitive paper moving at a pre-selected speed (New Electronic Products Ultra-violet recorder, type 1050). For the measurement of the force transmitted to the lower punch, the six upper threads of the lower punch holder were removed. Three strain

gauges were bonded to the holder and connected in series (see Fig. 1). A compensating resistance was constructed to complete the second recording channel. The system was calibrated by placing the upper punch in the lower punch holder. The assembly was then mounted on three previously standardised strain columns and stressed by means of a hydraulic press, care being taken to maintain axial loading. A linear relation between galvanometer deflection and applied force was found. The deflection was calibrated by introducing a resistance into the bridge equivalent to a 0·1 per cent out-of-balance. This corresponded to 1,100 kg. for the upper punch and 3,015 kg. for the lower punch, and varied less than 0·3 per cent in three independent trials.

The Estimation of the Resistance to Crushing

The Monsanto Hardness Tester and the Strong-Cobb Hardness Tester are commonly used to estimate the hardness of tablets. Both suffer from

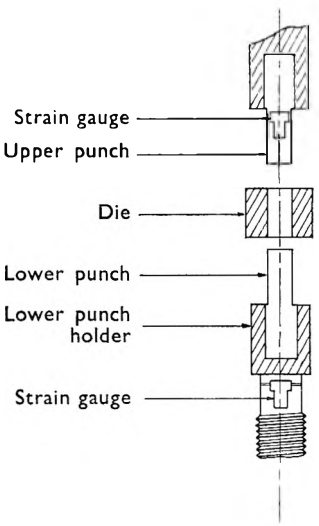

FIG. 1. Instrumented punch assembly.

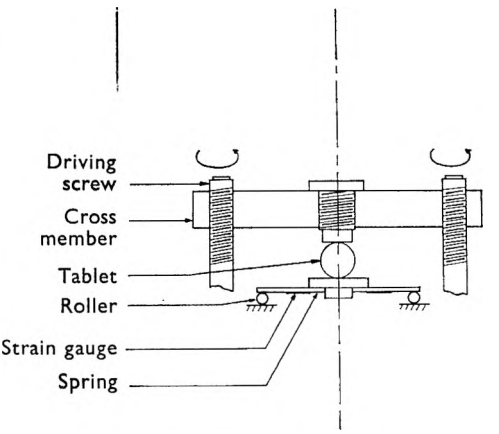

FIG. 2. Apparatus for measuring the resistance of tablets to crushing.

the disadvantage that the rate of loading is not constant. For the present work, it was decided to retain the crushing mechanism of these tests for subsequent comparison but to design apparatus capable of loading the compact under test at a constant rate. This apparatus is diagramatically represented in Figure 2.

The spring was supported at either end by hardened steel rollers. Two strain gauges (Tinsley, Type 6K, gauge factor 2·15, resistance 100 ohms) were bonded to the underside of the spring, one on each side of the central platform, and connected in series. A compensating resistance completed a circuit similar to that already described. By means of a low geared motor, the driving screws were rotated, thus raising or lowering the cross member. The tablet was centrally placed, on edge, on the

platform. The cross member was then lowered against the action of the spring until the compact failed. Deflection of the spring caused elongation of the strain gauges and a proportional change in resistance.

The system was calibrated by loading the spring in known increments and recording the galvanometer deflection. The calibration curve was linear, a 0·1 per cent out-of-balance corresponding to 19·10 kg. The trace of deflection versus time was also linear, indicating a constant rate of loading equal to 1·6 kg. sec.$^{-1}$.

Experimental

Sodium chloride was carefully sieved and a 30–40 mesh fraction selected. The density was estimated and the moisture content determined. In the preparation of the tablets a constant weight of material was used, calculated to give a tablet of thickness 0·4 cm. at zero porosity.

TABLE I

Summary of the compressive forces in the preparation of tablets from sodium chloride

Upper punch pressure P_a kg./sq. cm.	Lower punch pressure P_b kg./sq. cm.	Mean compaction pressure P_m kg./sq. cm.	$\frac{P_b}{P_a}$	Ejection force F_e kg.
1,854	1,491	1,673	0·804	216
1,504	1,236	1,370	0·822	163
1,224	1,027	1,126	0·840	125
1,016	853	934	0·840	96
793	670	732	0·844	71
606	511	559	0·845	55
417	350	384	0·841	37

Each value given is the mean of twelve results

Tablets were prepared at a constant rate of 68 tablets per minute and at each pressure level feed conditions were allowed to stabilise by rejecting the first ten tablets. The succeeding twelve tablets were then collected in sequence and individually weighed. The thickness and diameter were measured with a micrometer and, finally, the crushing pressure determined.

In a typical compression cycle, the deflection of the galvanometer which remained after the upper punch had been withdrawn, represented the force remaining on the bottom punch due to the elastic recovery of the compact against the die wall. Over the range studied, this force was required to eject the tablet from the die.

Results and Discussion

Table I summarises the experiments carried out giving the pressure exerted by the upper punch (P_a), the pressure transmitted to the lower punch (P_b), and the force required to eject the tablet (F_e).

The ratio of the lower punch pressure and the upper punch pressure remains constant over the range 400–1,000 kg./sq. cm. The relation

between the mean compaction pressure ($P_m = (P_a + P_b)/_2$) and the ejection force (F_e) is linear over this region. (See Fig. 3.)

Within this range, it is probable that the directional distribution of forces remains the same, with a constant proportion being transmitted to the die wall due to an increase in the area in contact with the die wall proportional to the applied force. Above 1,000 kg./sq. cm., a higher fraction of the applied force was transmitted to the die wall as shown by a fall in the ratio of lower punch pressure and upper punch pressure resulting in an increase in the slope of the ejection force curve. This deviation could be explained either by an increase in the coefficient of friction of the compacting material with pressure, or alternatively, by flow

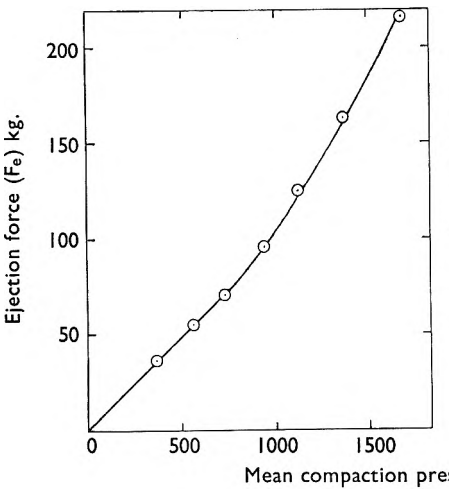

FIG. 3. Relation between the mean compaction pressure (P_m) and the ejection force (F_e).

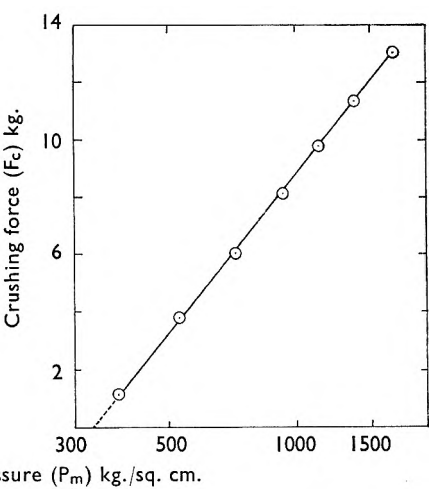

FIG. 4. Relation between the mean compaction pressure and the crushing strength of the tablet (F_c).

of material past the lower punch. This would give an increase in the area in contact with the die wall. There was, however, no visible evidence of flow as the edges of all tablets were clean and square.

The Strength of the Compacts

The mechanism of compact failure was complex. A wedge of material was formed in contact with the upper plate and the lower platform. The wedges eventually cleaved the tablet across the diameter, giving immediate relief of pressure as shown in Figure 5.

The relation between the mean compaction pressure (P_m) and the resistance to crushing of the compact (F_c) is shown in Figure 4.

The experimental results closely follow the relationship:

$$\log P_m = nF_c + C$$

where n and C are constants. Extrapolation of the line gives a value of C equal to 320 kg./sq. cm. and which represents a minimal pressure for the

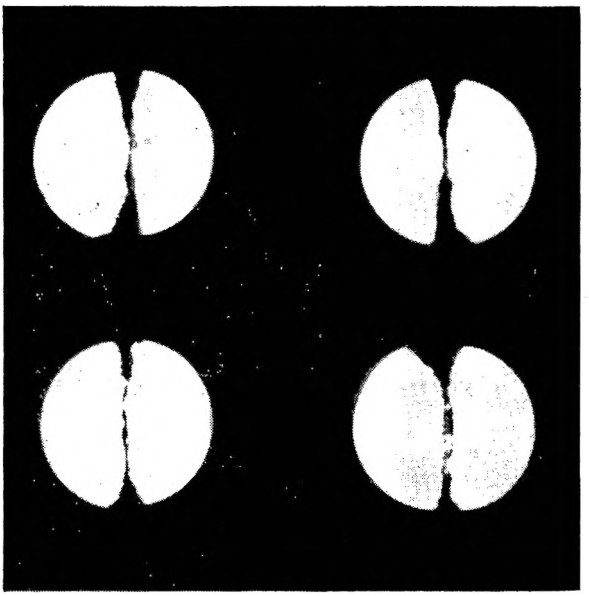

FIG. 5. Examples of the fracture of tablets.
Upper row: Tablets made under high compression.
Lower row: Tablets made under low compression.
A similar wedge formation is shown by both series.

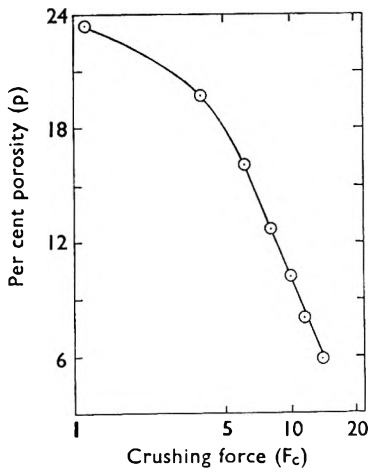

FIG. 6. The effect of porosity on the crushing strength of the tablet (F_c).

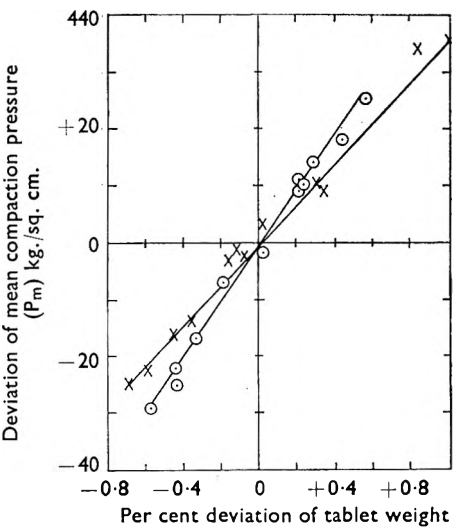

FIG. 7. The effect of variation of tablet weight on the mean compaction force (P_m).
X, P_m = 935 kg./sq. cm.
O, P_m = 1370 kg./sq. cm.

formation of a compact. Below this value, the compact should have no residual strength. Attempts to form such a compact failed.

Bal'shin[6] formulated the following relationship between the strength of a compact and the voidage for powdered metals and ceramics

$$S = S_o V_r^{-m}$$

where S is the strength of the compact (F_c), V_r is the relative volume, m is a constant and S_o is the strength when $V_r = 1$. The relative volume, V_r, is the ratio of the observed volume of the compact to the theoretical volume of material.

A further relationship was proposed by Ryshkewitch and Duckworth[7,8]

$$S = S_o e^{-bp}$$

where p is porosity and b is a constant.

Over the porosity range we studied, these equations are mathematically similar. The results for sodium chloride plotted according to the Ryshkewitch equation are given in Figure 6.

The results show excellent agreement over the range 5–16 per cent porosity. Extrapolation gives a value for the crushing strength of 22 kg. at zero porosity. Above 16 per cent porosity, the results show increasing deviation. The equation was derived from experiments on rigid porous materials and does not seem to apply to the loose compact produced at low pressures. At higher pressures, and certainly over the range of pressures used in the preparation of tablets, the equation would seem to accurately express the relation between strength and voidage.

The effect of variation in weight of fill. As with most tablet machines, the measure of die charge was made by volume. The inherent variation of weight which results from this method was reflected in the forces applied to the tablet. The variation of weight and of applied pressure is shown in Figure 7. Two series are shown (1,370 and 935 kg./ sq. cm.), and the deviation plotted for the twelve individual tablets of each series. Both series indicate that a variation of 0·5 per cent in weight produces a 2 per cent variation in the mean compaction pressure. By reference to Figure 4, this is equivalent to a 1 per cent variation in the resistance to crushing, discounting the effect of a slightly differing thickness.

Acknowledgements. The authors wish to thank Dr. D. Train for the use of the electronic apparatus which was bought with the aid of a grant from the Department of Scientific and Industrial Research. The D.S.I.R. is also to be thanked for a research studentship awarded to Mr. D. Ganderton. We also wish to thank the Distillers Company Ltd. for the basic apparatus for measuring crushing strength which we modified to our own requirements.

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THE STRENGTH OF COMPRESSED TABLETS

PART II. THE BONDING OF GRANULES DURING COMPRESSION

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The deformation of tablet granules has been followed over a wide pressure range by preparing and compressing sucrose granules with coloured surfaces. By examination of the fracture of the compacts in a strength test, it has been possible to determine the structural role of the granule at these pressures.

IN investigating the factors affecting the strength of a compressed tablet, it was considered desirable to construct a qualitative picture of the behaviour of tablet granules during compression and subsequent fracture.

Seelig and Wulff[1] divide the mechanism of the compression of powders into three stages. (*a*) Packing; during this stage, interparticular friction absorbs most of the applied energy. (*b*) Elastic and plastic deformation; the applied energy is mainly consumed as interparticular and die-wall friction. (*c*) Cold working, with or without fragmentation.

Although these hypotheses were devised to explain the compression of powdered metals, Seth[2] used them as an empirical account of the formation of pharmaceutical tablets, considering that fragmentation caused the initial increase in the total surface of the tablet reported by Higuchi, Rao, Busse and Swintosky[3]. This communication is a qualitative discussion of these factors.

A sharp demarcation of the granule boundary was obtained by Strickland, Nelson, Busse and Higuchi[4]. Granules were covered with activated charcoal in a successful attempt to determine the distribution of lubricant in compressed tablets.

In our work, observation of the boundary was made possible by colouring the outer layer of the granule.

EXPERIMENTAL

A roughly spherical sucrose granulation was prepared in a rotating coating pan. A small quantity of sucrose solution was added to crystal nuclei and the mixture dried. This process was repeated until an approximately 14–22 mesh material was obtained. This size was chosen because of its suitability for subsequent examination in the tablet matrix. The final addition of syrup contained a vivid dye, so that each granule received a thin coating of colour. Six batches, each of a different colour, were prepared and screened.

After determination of its density, the granulation was compressed on the instrumented tablet machine already described[5], and the compression forces recorded. Tablets were produced at five pressure levels with eight tablets in each series. A constant weight of fill was used, calculated to give a tablet 0·4 cm. thick at zero porosity. As sucrose exhibits high frictional effects when compressed, the punch and die surfaces were

lubricated with a solution of stearic acid in acetone and carbon tetrachloride. After measurement of weight and thickness, six tablets from each series were subjected to the crushing test previously described[5]. The two remaining tablets were used for examination by microscope. An upper surface and a fractured surface of a tablet from each series was photographed. Examples from two series are given in Figure 1.

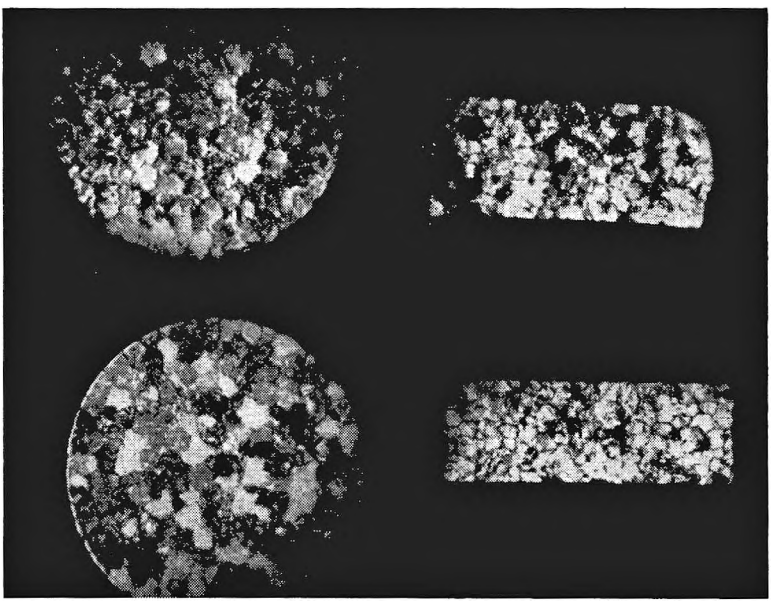

FIG. 1. Examples of tablet surfaces and fracture. The upper tablets were made at a mean compaction pressure of 530 kg./sq. cm. and the lower at 2,020 kg./sq. cm.

RESULTS

The values of mean compaction pressure, P_m, crushing force and porosity are given in Table I.

The relationship between porosity and the resistance to crushing is given in Figure 2. The results are plotted according to the equation:

$$F_c = F_{co}e^{-bp}$$

where F_c is the crushing force, F_{co} the crushing force at zero porosity, p is the porosity and b is a constant. This equation was empirically derived by Ryshkewitch and Duckworth[6] for porous ceramic materials and has been found applicable to the compaction of some drugs.

At low pressure (530 kg./sq. cm.), examination of the upper surface still reveals the original spherical configuration of the granules and interstices. With increase in pressure, the granule was seen to be distorted massively, accommodating the changes in neighbouring granules, and producing a close interlocking network, with the elimination of the surface interstices. The surface of the granules shows slight white striations at low pressure indicating a small amount of surface cleavage which

reveals the uncoloured material of the bulk of the granule. This effect increases progressively with increase of pressure until the surface layer was seen as coloured fragments on a white background.

TABLE I

MEAN COMPACTION PRESSURE, CRUSHING FORCE AND POROSITY

Mean compaction pressure P_m kg./sq. cm.	Crushing force F_c kg.	Porosity p per cent
2,020	10·13	7·55
1,655	7·47	9·29
1,375	5·82	10·86
855	2·63	16·25
530	1·58	19·92

Mean values are quoted

Examination of the fractured surface also gave the opportunity to follow the interlocking progression but in a plane at right angles to the surface and parallel to the direction of the applied force.

The actual line of fracture in the strength test is of great importance. In the low pressure series, 530 kg./sq. cm., the line of fracture usually

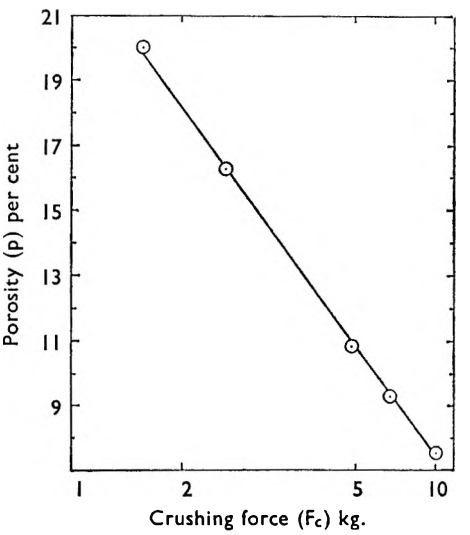

FIG. 2. The relation between porosity and crushing force.

follows the boundary of the granules, and leaves a coloured surface for observation. At a pressure of 855 kg./sq. cm., the proportion of granule surface visible on the fractured surface is much smaller and the tendency to break across the granule predominates. At 1,375 kg./sq. cm. and above, this is the only mode of failure.

DISCUSSION

The stages of powder compression listed in the introduction will not be clearly defined but will overlap to an extent varying with the observed

material. The relation between the porosity and the crushing force is found to be linear over the observed range, suggesting that there is no sharp transition from one mode of behaviour to another. Also, it may be concluded that the decrease in voidage due to packing is largely complete in the tablets produced at even the lowest pressures. These tablets exhibit a porosity of under 20 per cent whereas the minimum porosity without deformation for a material of this size range and shape is over 25 per cent.

Examination of the fractured surfaces indicates the pressure range over which the structural identity of the granule is lost due to fragmentation. Before this, failure occurs mainly around the granule. This is shown in the series at 530 and to a lesser extent at 855 kg./sq. cm. where the strength of the intergranular bond is increasing. Fracture across the granule indicates that the bond between adjacent particles of different granules is at least as strong as the bonding forces in the granule. Over this range, therefore, there must have been considerable fragmentation and rebonding of the fresh surfaces as suggested by Higuchi's report[3] of an increase in the total surface area, followed by a decrease, during compression. Where the fractured surface shows that cleavage has taken place independently of the original granule configuration, fragmentation and rebonding has destroyed the original structural units. In view of this, it is doubtful if the apparent interlocking in which each granule participates on compression has the structural significance found in the compression of some of the dendritic materials used in powder metallurgy and suggested as a mechanism in the bonding of tablets by Seth.

The minimum porosity obtained was 7·55 per cent although no voids are apparent in a microscopic examination. This also, is suggestive of a process of fragmentation and rebonding during which the void spaces found in the loose aggregate of granules (Series 1 and 2) are redistributed as a fine network (Series 3, 4 and 5).

Acknowledgements. The authors wish to thank Mr. D. R. Felstead of Barratt and Co. Ltd. for the supply of the coloured sucrose granules. This work was carried out with the aid (to D.G.) of a D.S.I.R. Research Studentship.

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After Mr. Ganderton presented the papers there was a DISCUSSION. The following points arose.

The work had not yet included the use of porous granules because initially it was intended to keep the system simple; the work should be continued on a rotary machine because of the different mode of compression.

THE USE OF LAMINAR LUBRICANTS IN COMPACTION PROCESSES

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Received May 20, 1960

A punch penetration shear test has been used to support the theory of orientation of layers within the crystal lattice as the lubricating mechanism of laminar solids. Under high applied loads, this orientation is prevented, thereby causing a high value of the shear strength. The increased shear strength is manifest in an increased coefficient of friction under high loads, as found experimentally in the die pressing of solid plugs of talc and graphite. The use of these solids as lubricants in compaction processes cannot be justified at high constraining loads, where the relative density of the compact is high.

Talc has always enjoyed the reputation of being a lubricant in pharmacy, and materials such as graphite and molybdenum disulphide have considerable practical importance as lubricants in many industrial processes. The common property of these materials is that they are laminar solids. Their mechanism of lubrication is frequently attributed to loosely bound lattice layers of the solid sliding easily over each other when placed between moving surfaces[1] (Fig. 1*a*). However, other work[2,3] has also emphasised the importance of the presence of adsorbed materials such as water, ammonia or acetone, since complete degassing produces a material in which the coefficient of friction is extremley high.

Fullam and Savage[4] have shown that by alternating the direction of the moving surfaces a very high frictional coefficient could be obtained. They suggested that when graphite acts as a lubricant, it forms layers

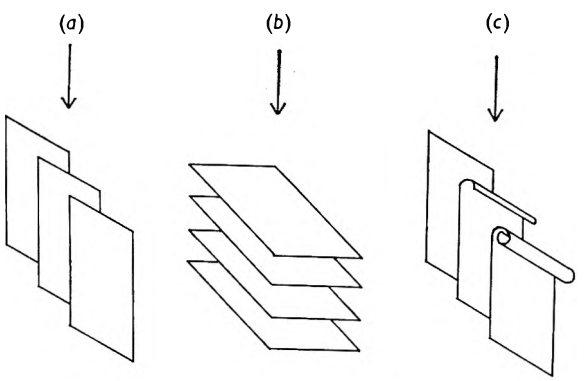

Fig. 1. The mechanism of lubrication of laminar solids.
(*a*) Slip of laminar plates over one another
(*b*) the orientation of plates at 45°, and
(*c*) the "roller bearing" action.

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which are orientated at about 45° to the moving surface. Subsequently, on alteration of the direction of motion, these layers oppose movement until a reorientation within the lattice had been achieved. (Fig. 1*b*.)

Recent work[5] involving the electron microscope has indicated that layers of laminar lubricants roll up in the direction of motion, to form a

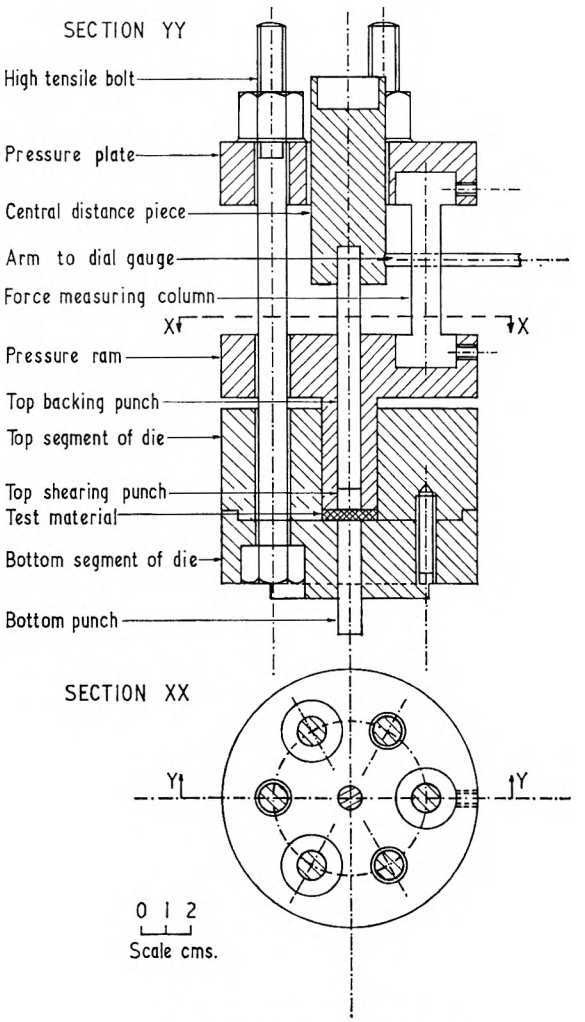

FIG. 2. Shear strength apparatus.

structure which, in the case of graphite, is similar to "whiskers" of the same material[6] (Fig. 1*c*). It is suggested that these rollers could be produced only if there is a loosening, for example, by the presence of water vapour, of the inter-layer binding forces, first at the edges of the crystals and then, later, inside them. This roller mechanism would also explain the high coefficient obtained on alternating the direction of

motion of the sliding surfaces. In these circumstances the rollers would have to be unravelled and reformed in the opposite direction.

It is also realised that for the roller mechanism to act efficiently, sufficient space for the roll to form must be available between the sliding surfaces. High pressures, such as are found in the compaction of a tablet mass, would be likely to restrict this mechanism and the purpose

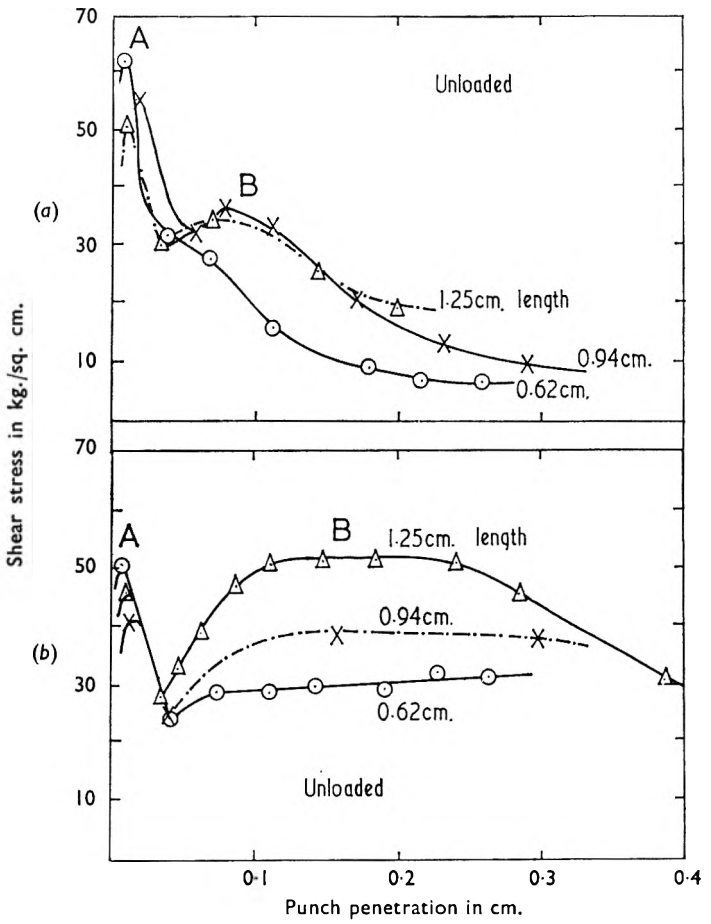

FIG. 3. Shear strength determination of talc. The lengths of plugs are given.
(*a*) In direction normal to the grain
(*b*) in direction parallel with the grain.

of this work was to examine the shear properties of such lubricants for evidence to support this postulate.

APPARATUS

The apparatus used to measure the shear strength of talc and graphite (Fig. 2) operates on a punch penetration principle similar to that used

in the blanking process[7]. However, using this apparatus the shear properties of specimens may be measured under high axial loads. The apparatus consisted of a horizontally split die, the upper segment of which has a bore of diameter equal to that of the sample, whilst the lower segment has a bore accommodating only the lower punch. Three

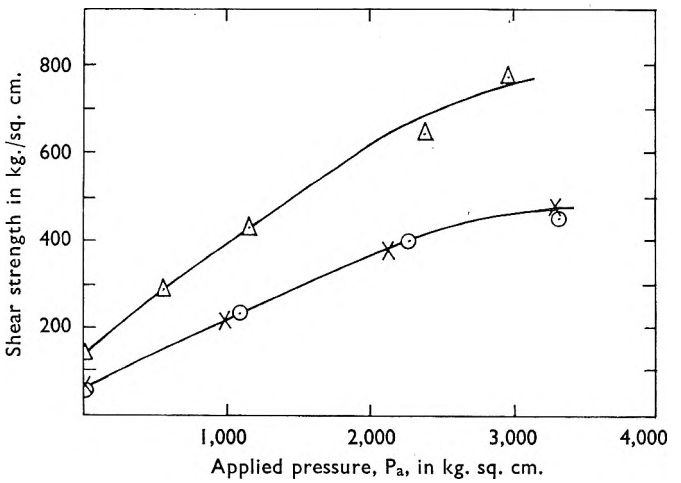

FIG. 4. Effect of applied load on the shear strength of graphite and talc.
△ Graphite.
× Talc (parallel with grain).
⊙ Talc (normal to grain).

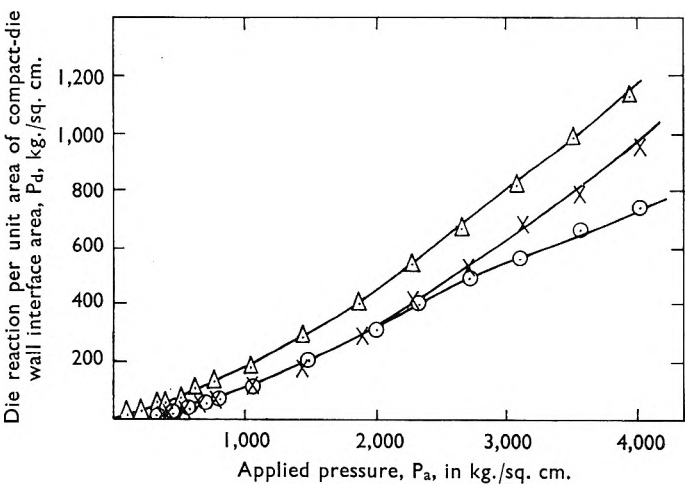

FIG. 5. Die wall friction using solid talc plugs. Die-bore 2·42 cm. diameter.
△ Diameter 2·43 cm., both with and across the direction of the grain.
⊙ Diameter 2·40 cm., across the direction of the grain.
× Diameter 2·30 cm., in the direction of the grain.

high tensile bolts were used to give a force on the pressure plate, which is transmitted, by means of three force measuring columns, to the pressure ram. The apparatus was machined from hardened Al3 steel (Edgar Allen and Co. Ltd.).

The general apparatus and its operation has been previously described by Train and Carrington[8]. The "moving die" technique described by these authors was used throughout. The shear strength apparatus (Fig. 2) replaces the normal punch and die assembly of the die pressing apparatus between the platens of a hydraulic press.

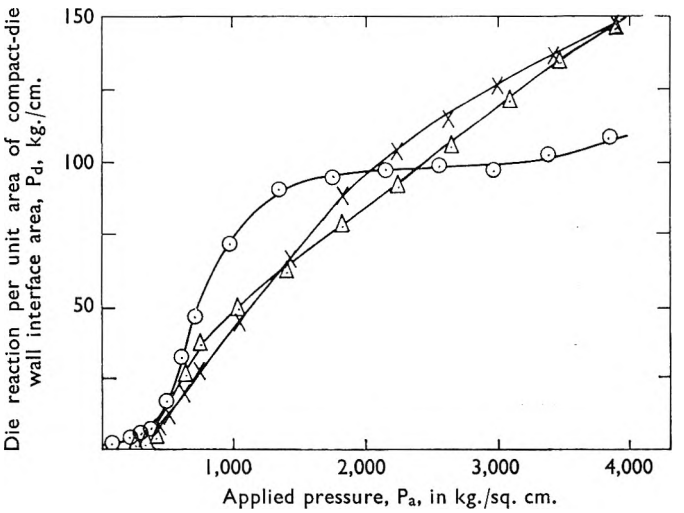

FIG. 6. Die wall friction using lubricated graphite plugs.
× Unlubricated.
△ Lubricated with colloidal graphite.
○ Lubricated with lead foil.

EXPERIMENTAL

The shear strength of graphite (grade EY4A, Morgan Crucibles Ltd.) and of talc, natural crystal, in the directions normal and parallel to the direction of the grain was examined at pre-selected pressures. The samples were degreased using a solution of 50 per cent acetone and 50 per cent carbon tetrachloride. The mechanism of shear used in these experiments is that of forcing an outer ring of the cylindrical sample past a stationary central portion, using a secondary hydraulic ram. The shear forces were measured using the die supporting load cells at increments of punch penetration, indicated by displacement gauges.

The shear strength at zero applied pressure was determined by supporting the bottom punch on a simple helical spring. The pressure ram (Fig. 2) was then adjusted to hold the sample in position without applying a positive compressive load. The assembled apparatus was then placed on the die platform in place of the die-set of the diepressing apparatus, and a force measuring column was used in the shearing punch train.

In the apparatus to measure the shear strength of materials under load, the helical spring was replaced by a hardened A13 steel distance piece. The three high tensile bolts were tightened to a predetermined load over the outer circumference of the specimen, whilst the central punch train was adjusted to an identical pressure over the centre of the sample. The results are presented in Figures 3 and 4. Talc and graphite plugs and powders were also pressed in the die pressing apparatus. The die wall friction of an unlubricated graphite plug was also compared with one lubricated with "Acheson" colloidal graphite and another contained in a wrapping of lead foil. These results are given in Figures 5 to 8.

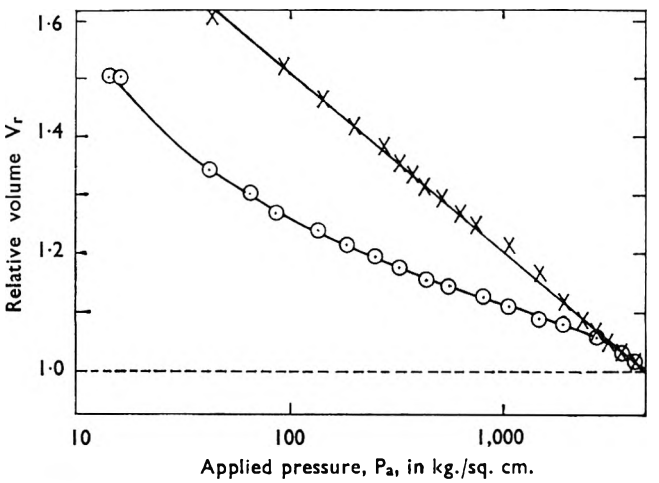

FIG. 7. Consolidation of talc and graphite powders.
× 4·0 g. graphite powder.
⊙ 11·6 g. talc powder.

DISCUSSION

The shear curve of talc (Fig. 3) shows an interesting phenomenon under zero load. As punch penetration proceeds the shear stress is built up until the sample shears at a relatively early extent of punch penetration, A. Subsequent penetration results in a further build up of shear (or frictional) resistance until a second maximum is obtained, B. The phenomenon of a second maximum would appear to be a characteristic of anisotropic solids, and cannot be explained on a simple basis of shear. However, the orientation of layers within the crystal lattice (Fig. 1*b*, or *c*) subsequent to shearing could explain this build up of frictional resistance. Graphite also shows this phenomenon.

Thus, the shear strength of talc given by A shows that the value in the direction of the grain is within 15 per cent of that across the grain. This indicates that a simple shear mechanism does not operate, when it would be expected that the shear strength in the direction of the grain would be much less than that across it.

Figure 4 traces the relationship of the shear strength of talc and graphite against load. The considerable increase of strength of these

materials under applied load indicates that the constraining load on the sample prevents orientation within the crystal lattice, resulting in a high shear strength value which is consistent with a high coefficient of friction[1].

A theoretical analysis of the die wall fraction has shown the importance of the shear strength in evaluating the stress distribution within a compact when compressed in a die[9]. The die pressing of talc plugs with a slip fit (Fig. 5, 2·40 cm. dia.) shows that above an applied pressure of approximately 400 kg./cm.^{2} reorientation within the lattice is prevented, having the effect of an increasing die reaction attributed to the increased value of the shear strength.

Plugs injected into the die show (Fig. 5, 2·43 cm. dia.) a gradually increasing die reaction, since under these conditions, the layers cannot

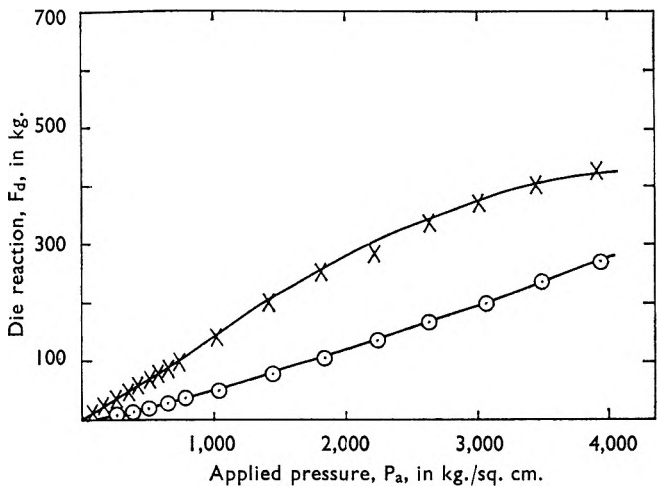

FIG. 8. Die wall friction using talc and graphite powders.
× 4·0 g. graphite powder.
○ 11·6 g. talc powder.

reorientate even at low applied axial thrusts as they are constrained by lateral forces. The pressing of graphite plugs wrapped in lead foil (Fig. 6) shows that under high axial loads (above 2,400 kg./cm.^{2}) the lead foil, which shears preferentially compared with the graphite, is the more effective lubricant.

However, with powder systems of talc and graphite the relative volume[10] of the systems is very high (Fig. 7) and decreasing only gradually under compacting forces. Under conditions of high voidage, sufficient space is available for the laminar solid to orientate or form rolls, thereby giving reasonable lubrication. The die reaction observed in the pressing of these powders is correspondingly low.

CONCLUSIONS

The shear strength determination of graphite and talc suggests that the mechanism of lubrication of these solids arises from some orientation within the crystal lattice, probably involving the roller bearing action.

Under high constraining loads, this orientation or rolling up of the layers of the laminar solid is prevented, thereby causing high values for both the shear strength and coefficient of friction.

These materials will act as efficient lubricants only in compaction processes when the voidage of the compact is high. Talc and graphite should not be used as lubricants in the compaction of materials which will deform at low applied pressures to form a solid compact.

Acknowledgements. We wish to express our gratitude to B.D.H. Ltd. for the gift of the natural crystalline talc; to Mr. C. Shears for machining the talc and graphite specimens, and to Mr. J. J. Deer for his help with the assembly of the apparatus.

This work was carried out with the aid (to J. A. H.) of the Burrough's Scholarship and a D.S.I.R. Research Studentship.

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After Dr. Hersey presented the paper there was a DISCUSSION.

THE EMULSIFYING PROPERTIES OF GUM ACACIA

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Received May 23, 1960

The quantity of potassium arabate required to stabilise as an emulsion a known volume of a hydrocarbon oil has been determined and an estimate of the dimensions of the interfacial film made. The gum acacia remaining in the washed emulsion is held strongly at the oil: water interface and is not yielded to the bulk of the aqueous phase.

An attempt was made to determine whether acacia is adsorbed at the oil water interface in equilibrium with the bulk solution or if it is irreversibly adsorbed. If it is in equilibrium repeated washing of the emulsion should cause the destruction of the emulsion as the gum is removed from the system.

Method

Emulsions of light liquid paraffin, benzene, cyclohexane and heptane were made by mixing equal volumes of hydrocarbon oil and approximately 10 per cent w/v solution of potassium arabate, stirring with a dispersator and then passing twice through a hand homogeniser.

Washing of the Emulsion

The method used was an adaption of that described by King[1]. A convenient volume of an emulsion was diluted to twice its volume with water and centrifuged to separate the phases. Nearly all the separated aqueous phase was removed and replaced by a similar volume of water, and after thorough mixing the emulsion was centrifuged again. This process was repeated for as many cycles as necessary.

A heptane emulsion was examined after seven such cycles and the oil content was determined by comparison of the densities of oil, water and emulsion. The surface area of the globules was calculated by measuring diameters and counting the numbers of droplets on twenty photomicrographs. The distribution of gum between the aqueous phase and the interface was found by evaporation of samples of aqueous phase and emulsion respectively.

Results

Benzene emulsions could be washed several times without obvious change but after the fourth cycle the emulsion began to crack, and thereafter each washing yielded a small volume of benzene. Cyclohexane emulsions were more stable, no sign of cracking appeared until the seventh washing, but even after the fourteenth the bulk of the emulsion remained. A heptane emulsion did not show any sign of deterioration after the seventh washing (see Table I), and a light liquid paraffin emulsion was stable after ten washing cycles. All these washed emulsions stored well but in most there was a noticeable coarsening after several weeks. The stability appears to be the same order as the resistance to washing.

Discussion

In an emulsion which has been washed until the concentration of potassium arabate in the continuous phase is virtually zero it is reasonable to suppose that the amount of gum remaining is the minimum quantity necessary to stabilise that emulsion. There is experimental evidence for the interfacial area of the oil globules, the weight, and hence the number of molecules of potassium arabate adsorbed. To calculate the thickness of, and number of layers of molecules in the interfacial film, we need to know the volume and shape of the molecule. The conclusions of Veis and Eggenberger[3] that the arabic acid molecule is a short stiff coil, of length slightly greater than its diameter does not seriously disagree with those of Matthews[4] who described the molecule as spherical, but the estimates of size cannot be reconciled. Matthews' estimate must be discounted since it was based on over-simplified structure construction of the molecule

TABLE I

Composition of a heptane emulsion after seven washing cycles

Volume composition (from density measurements)	23·0 per cent v/v
Volume of heptane in 50 ml. of emulsion	11·6 ml.
Weight of gum in 50 ml. of emulsion	0·0672 g.
Weight of gum in 50 ml. of aqueous phase	not weighable
Weight of gum per ml. of emulsion	1·4 mg.
Weight of gum per ml. of heptane	5·8 mg.
Interfacial area of heptane in 50 ml. emulsion	$9{\cdot}55 \times 10^{20}$ Å^2
Number of molecules of potassium arabate (mol. wt. 3×10^5)^2	$1{\cdot}35 \times 10^{17}$
Area per molecule	$7{\cdot}07 \times 10^3$ Å^2
Number of molecules of potassium arabate (mol. wt. 10^6)^2	$4{\cdot}049 \times 10^{16}$
Area per molecule	$2{\cdot}35 \times 10^4$ Å^2

whereas that of Veis and Eggenberger for molecules of molecular weight 10^6 and at limiting concentration represents a molecule at its maximum size. That the volume of $7{\cdot}2 \times 10^{-16}$ ml. proposed by Veis and Eggenberger is too large for real solutions is shown by their statement that solutions of independent non-overlapping molecules of that site cannot exist above concentrations of 2×10^{-3} g./ml.

If the concept of an isodiametric molecule be accepted it becomes possible to speculate about the dimensions of the interfacial film in the heptane emulsion. From the measured area per molecule, the film thickness is derived, and since the weight of gum is known a volume and concentration can be assigned to the interfacial film. Table II shows the results of calculations based on acacia solutions of various strengths.

One case, that of the 65 per cent w/v solution, gum of molecular weight 3×10^5, fits the required dimensions of a monolayer of an isodiametric molecule very well. A solution of this concentration is an elastic gel. The remaining examples although fitting the requirements much less satisfactorily, do tend to support the hypothesis of a monolayer interfacial film.

During this work, the stability of the washed emulsion was very noticeable, even when heated to about 105° to volatilise both phases, the emulsion did not break until the drying was nearly complete. The potassium arabate recovered from the emulsion in this way was readily soluble in

water and did not appear to have been denatured. The mechanism by which the molecule of potassium arabate becomes so securely adsorbed at the interface in the apparent absence of a lipohilic group remains obscure but it seems the mechanism will be different to that of soaps, proteins and saponin because these either contain a lipohilic group or denature during use.

This interfacial film of acacia cannot be removed by very considerable dilution of the aqueous phase which seems to indicate the adsorption of this monolayer is not an equilibrium process such as envisaged by Gibbs.

TABLE II

RESULTS OF CALCULATIONS BASED ON ACACIA SOLUTIONS OF VARIOUS STRENGTHS

1	2	3	4	5	6	7	8
Molecular weight 3×10^5	State of acacia solution per cent w/w or solid	Vol mol calculated ($Å^3$)	Area mol measured ($Å^2$)	Film thickness from $\frac{\text{col. 3}}{\text{col. 4}}$ calculated Å	Film thickness from $\sqrt[3]{\frac{\text{vol}}{\text{mol}}}$ calculated Å	Fit to measured area $\frac{(\text{Col. 6})^2}{\text{Col. 4}}$	Fit to calculated thickness $\frac{\text{Col. 5}}{\text{Col. 6}}$
	Solid	$0{\cdot}33 \times 10^6$	$7{\cdot}0767 \times 10^3 = (84{\cdot}12)^2$	46·9	69·24	0·675	0·825
	75	$0{\cdot}498 \times 10^6$	"	70·3	78·9	0·88	0·837
	65	$0{\cdot}5928 \times 10^6$	"	84	84	1·00	1·00
	50	$0{\cdot}826 \times 10^6$	"	116·72	93·82	1·24	1·39
	33·33 O:W:G 4:2:1	$1{\cdot}494 \times 10^6$	"	211·2	114·4	1·84	2·50
10^6	Solid	$0{\cdot}11065 \times 10^6$	$2{\cdot}356 \times 10^4 = (153{\cdot}5)^2$	46·9	47·9	0·098	0·313
	75	$0{\cdot}166 \times 10^7$	"	70·3	118·5	0·21	0·775
	65	$0{\cdot}2 \times 10^7$	"	84	126	0·3	0·824
	50	$0{\cdot}2755 \times 10^7$	"	116·8	140·2	0·582	0·916
	33·33	$0{\cdot}4978 \times 10^7$	"	211	170·8	1·89	1·115

Such a monolayer whilst sufficient to stabilise an emulsion may form a multilayer when in equilibrium with an acacia solution as used in emulsions. The substantial nature of these thick films is indicated by the behaviour of droplets of acacia solutions in oils[5].

The varying degree of stability of the different emulsions to the washing process indicates that the efficiency with which the gum is adsorbed depends at least in part on the nature of the oil.

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After Mr. Wibberley presented the paper there was a DISCUSSION. The following points arose.

After seven washings of a heptane emulsion, the acacia remaining could be presumed to be the minimum amount required to stabilise the emulsion as it existed. If this emulsion were to be subjected to a further homogenisation then it would probably crack because there would be insufficient acacia to stabilise the fresh interfacial area produced.

RHEOLOGY OF ACACIA-STABILISED EMULSIONS

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Received May 18, 1960

The flow behaviour of emulsions of benzene and two paraffin oils in acacia solutions has been examined. The concentration of acacia had little effect on the relative viscosity, and the viscosity of the oil none. Emulsion viscosity increased with the volume fraction of oil which also led to an increased deviation from Newtonian flow. Evidence of thixotropy was not found in these emulsions. The chemical nature of the oil has a great influence on the viscosity and the differences are explained in terms of the characteristics of the interfacial film.

The rheology of emulsions containing moderate concentrations of oil has been studied by Richardson[1], Sibree[2], Toms[3], Neogy and Ghosh[4], and others[5,6]. General agreement had been found on the effect of increasing oil concentration, but differences were noted when the nature of the stabiliser or oil were changed. No work of this kind has previously been undertaken using a hydrophilic colloid as emulsifying agent. Sumner[6] has pointed out the need to investigate the viscosity of emulsions stabilised by agents such as acacia, which are known to produce a visible film at the oil-water interface[7].

Experimental

Apparatus. A Ferranti-Shirley cone-and-plate viscometer was used. This has been described in detail by McKennell[8]. The original torsion spring was replaced by a lighter one so that a full scale deflection at maximum sensitivity was obtained with a fluid of about two poises. The temperature throughout the work was approximately 25° maintained by circulating water at 25 $\pm$ 0·1° beneath the plate.

A 1 in. diameter "Dispersator" head (Premier Colloid Mills) was used on a Mitchell laboratory stirrer for making the emulsions.

Materials

Potassium arabate was produced from acacia by repeated precipitation with ethanol from an aqueous solution followed by passage through an ion exchange column to produce free arabic acid. The potassium salt was made by adding Analar potassium hydroxide to the acid in solution until the pH rose to 7·0. The method was described by Shotton[9]; larger batches (500 g.) were prepared and the salt was precipitated with ethanol before drying under vacuum.

The benzene was of Analar quality. Liquid Paraffin B.P., and Light Liquid Paraffin B.P. were used without further purification. They were free from acid and readily carbonisable materials, and contained no unsaturated substances. They had the following physical characteristics.

	Viscosity poises	Density g./ml.
Liquid Paraffin B.P.	1·5280	0 8796
Light Liquid Paraffin B.P.	0·2910	0 8483

Method

Emulsion preparation. A solution of potassium arabate and the oil were weighed into a 250 ml. beaker and stirred for 60 seconds at about 3,000 r.p.m. Two glass rods were held against the side of the beaker to act as baffles. Emulsions of a higher volume fraction than 0·45 were made by first preparing an emulsion containing 30 per cent of oil. The remaining oil was then added in small quantities until the required volume fraction was reached. Fine emulsions were made by passing the coarse one five times through a small hand homogeniser with the valve screwed up tight.

100 ml. volumes of emulsions of each of the oils were prepared at 0·1, 0·2, 0·3, 0·4, 0·5 and 0·6, volume fraction. Gum concentrations in the aqueous phase were 9, 10, 15, and 20 per cent w/v.

The emulsions were stored in small glass stoppered Pyrex glass bottles and the viscosity was measured after 4 days. After this period the entrapped air had been released and the interface was known to be in equilibrium[9].

Three emulsions were made for each concentration of oil and gum and three flow curves were produced for each emulsion. The mean of the nine results was taken.

Before placing in the viscometer, the emulsions were remixed by carefully rotating the containers for about 3 minutes. They were then homogeneous and substantially free from air. When a diluted emulsion was examined microscopically no aggregates of oil globules could be seen. The flow curve was quickly taken by raising the shear rate in uniform steps from 0 to 1,691 sec.$^{-1}$, and the shear stress read from the indicator. Sample points were observed on the down curve to investigate the possibility of thixotropy.

Results

Effect of Concentration of Potassium Arabate in the Aqueous Phase on the Relative Viscosity of Benzene Emulsions

Nine and 10 per cent potassium arabate solutions produced emulsions that were not so stable as those made with higher concentrations. Creaming was rapid and some large globules separated. On attempting to make emulsions of high oil concentration with a 20 per cent solution of potassium arabate cracking occurred. A 15 per cent solution produced stable benzene emulsions when the volume fraction varied from 0·1 to 0·6, and this concentration of potassium arabate was chosen for all subsequent work.

Small variations in relative viscosity were found for emulsions of the same volume fraction prepared with potassium arabate solutions of different concentrations.

Effect of Volume Concentration of Disperse Phase, and the Nature of the Oil used on Flow Behaviour

The flow curves were linear for a volume fraction up to between 0·2 to 0·3, but the emulsions were non-Newtonian when the oil concentration was increased above this value (Fig. 1). This deviation increased with

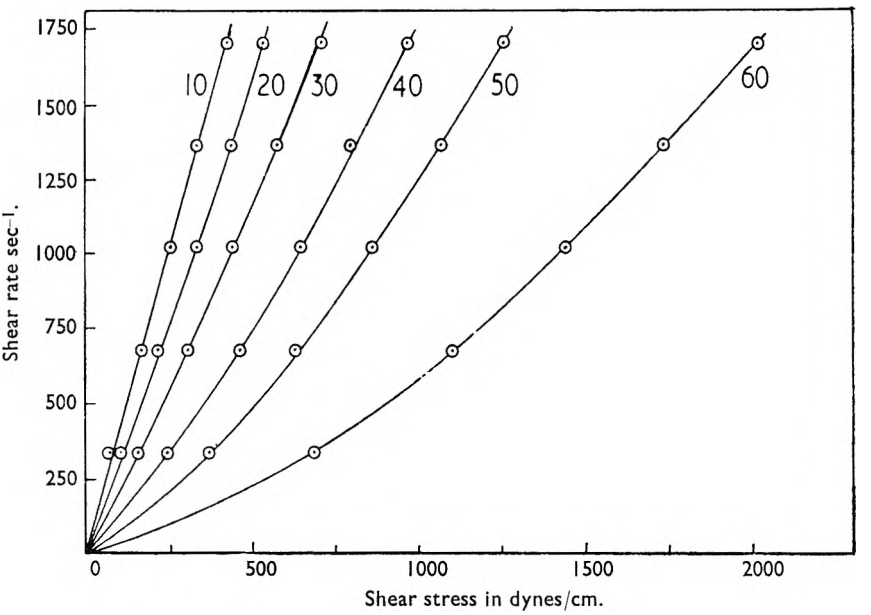

FIG. 1. Flow curves of benzene-potassium arabate (15 per cent w/v) emulsions. Figures are per cent volume concentration of oil.

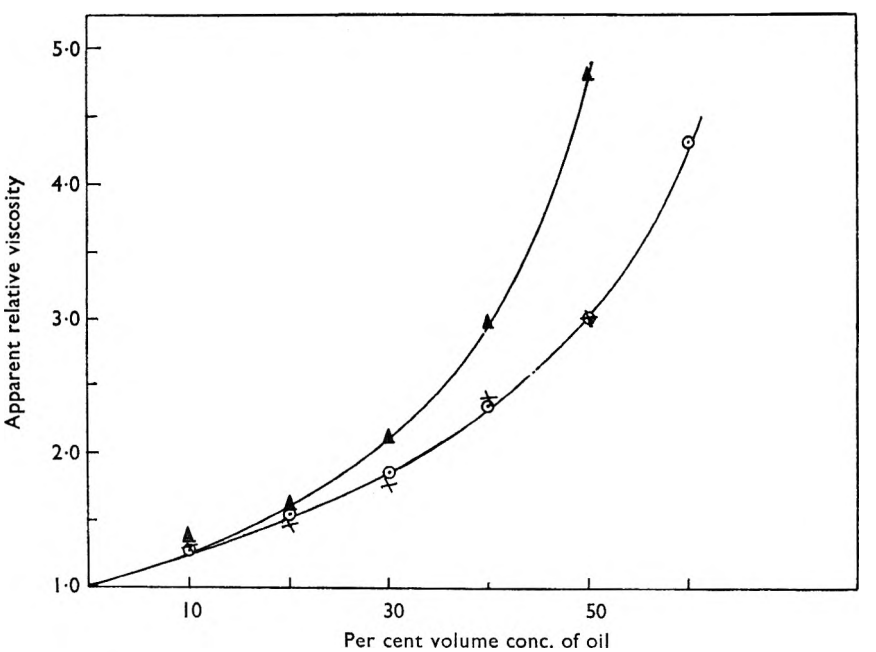

FIG. 2. Graph of apparent relative viscosity against volume concentration of oil.
▲ Light liquid paraffin X Liquid paraffin ○ Benzene

increasing oil concentration. No evidence of thixotropy was found even at the highest volume fraction.

Attempts have been made to represent the whole flow curve mathematically either by plotting log flow characteristics[10] or by using the Ree-Eyring equation for generalised flow[11]. However, in this instance, for comparison purposes, the apparent viscosity has been calculated from the linear portion of the plot in the manner reported by Sibree[2], Richardson[1] and Broughton and Squires[5].

A plot of apparent relative viscosity against volume concentration is almost linear to about 30 per cent volume concentration. Above this value the viscosity increases more rapidly with increase in the volume fraction as shown in Figure 2.

Benzene and liquid paraffin emulsions were similar in behaviour but light liquid paraffin emulsions were more viscous overall at any given volume fraction.

Homogenisation

Homogenisation of a benzene emulsion readily caused cracking, although with care, a fine emulsion could be made. The viscosity increased only slightly. A light liquid paraffin emulsion could be homogenised without difficulty resulting in a greatly increased viscosity.

Discussion

The flow behaviour of acacia-stabilised emulsions is similar to that of emulsions prepared with synthetic stabilisers. The emulsion viscosity rises with an increase in oil concentration, and this in turn is accompanied by a greater deviation from Newtonian flow.

It appears that the viscosity of the oil has no marked effect on emulsion viscosity, a conclusion reported by others[2,5]. However, with acacia-stabilised emulsions the nature of the oil does influence the viscosity of the emulsion.

It may be that the droplet size distribution varies from an emulsion prepared with one oil to that prepared with another, but both Sibree[2] and Leviton and Leighton[12] found that a change in globule size did not alter emulsion viscosity.

To explain the difference in the viscosities of emulsions of the same volume fraction but of different oils, it is postulated that a substantial film is built up at the oil-acacia solution interface, and that the nature of the film depends upon the oil used. A film of substantial thickness would increase the effective volume of the globules. There would, therefore, be an increase in volume fraction, and a corresponding increase in emulsion viscosity. Reduction in mean particle size would lead to an increase in the interfacial area available for adsorbing the film. Thus, as the mean particle size is reduced, the part of the volume fraction contributed by the film would increase. A raised viscosity would follow from the effective increase in volume fraction. Homogenisation should, therefore, cause an increase in the viscosity of emulsions where the globules are surrounded by a thick interfacial film.

Shotton and Wibberley[13] have shown that acacia quickly builds up a multilayer at the interface, and Serrallach and Jones[7] found in 1931 that a film visible to the eye was formed after 4 days. Serrallach, Jones and Owen[14] further discovered that the chemical nature of the oil used influenced both the physical appearance and the mechanical strength of the film at the oil-acacia solution interface.

It is suggested that benzene droplets are surrounded by a comparatively weak, thin film, and light liquid paraffin droplets by one which is thicker and stronger.

Such a difference in the nature of the film would explain why benzene emulsions were less viscous than those of light liquid paraffin at the same volume concentration. It would further explain the ease of cracking of benzene emulsions when homogenisation was attempted, and the small rise in viscosity when the particle size was successfully reduced. The thicker film surrounding light liquid paraffin droplets would account for the much increased viscosity on homogenisation.

The difference in viscosity between emulsions of the two paraffin oils is probably due to a smaller mean particle size of the light liquid paraffin emulsion.

Work to test this theory is being continued on the effect of the nature of the oil, the drop size distribution, and the age of the emulsion on flow behaviour.

Acknowledgements. This work was carried out with the aid (to R.F.W.) of an Educational Award from the Pharmaceutical Society of Great Britain.

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After Mr. White presented the paper there was a DISCUSSION. The following points were made.

The systems under investigation behaved as Newtonian fluids, and in spite of the high shear rates used there was no hysteresis in the experimental curves.

A NOTE ON THE STABILITY OF SOLUTIONS OF PHENYLEPHRINE

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Received May 4, 1960

Solutions containing phenylephrine hydrochloride (10 per cent) are commonly used as eye-drops to produce either a rapid dilatation of the pupil without loss of accommodation or a rapid and temporary reduction in the intraocular pressure in glaucoma. There appears to be no reference in the literature to the stability of such solutions although they may develop a yellow, pink or purple colour on storage.

In 1951, Schou and Rhodes[1] showed that injections of phenylephrine (1 per cent) are stable if made oxygen-free and strongly acidic (pH 1). They stated that sodium metabisulphite (0·1 per cent) must be added to solutions of a higher pH value to prevent coloration and loss of activity on autoclaving. Wahlquist[2] also noted that injections of phenylephrine become yellow on storage if sodium metabisulphite is omitted and oxygen is not totally replaced by nitrogen.

It was noted in hospital practice that solutions of phenylephrine (10 per cent) became yellow or pink within a few weeks of issue in 15 ml. amber-coloured eye-drop bottles, and sodium metabisulphite (0·1 or 0·2 per cent) did not prevent the discoloration. Although colour changes are not necessarily a guide to changes in biological activity attempts have been made to prepare solutions which would remain colourless.

The chelating agent, disodium edetate, was tested as it has been found effective in preventing coloration of solutions of procaine hydrochloride. (Green and Whittet, unpublished).

The solutions prepared, using "Solution for Eye-Drops B.P.C." as solvent, were: phenylephrine (10 per cent) alone, with 0·1 or 0·2 per cent sodium metabisulphite, with 0·1 per cent disodium edetate, or with 0·1 per cent disodium edetate and 0·1 or 0·2 per cent sodium metabisulphite. Samples were placed in 1 ml. ampoules under nitrogen, in corked or screw-capped 30 ml. white glass bottles, and in 15 ml. amber-coloured dropper bottles. All the ampoules were filled with the solutions but only half of the bottles were filled; the remainder were half-filled. All samples were then sterilised by heating with a bactericide at 100° for 30 minutes. They were examined at frequent intervals during nine months' storage at room temperature, and coloured solutions were biologically assayed on the blood pressure of the anaesthetised rat or cat.

The solutions in the ampoules, with the exception of those without added preservative, remained colourless throughout. Those without preservative were a faint yellow. There was no loss of biological activity

in any of these solutions. The solutions in the three types of bottles showed that the appearance of colour was slowest in the full ones stored away from the light, and that disodium edetate exerted a protective action against direct oxidation whereas metabisulphite was unsatisfactory. The

TABLE I

THE EFFECT OF STORAGE AT ROOM TEMPERATURE ON THE COLOUR OF STEAMED SOLUTIONS OF PHENYLEPHRINE (10 PER CENT) IN THE PRESENCE OR THE ABSENCE OF A PRESERVATIVE IN HALF-FULL 15 ML. AMBER-COLOURED EYE-DROP BOTTLES

Preservative added	Storage period (months)			
	1	3	4	6
None	Faint yellow	Yellow, with black specks	Brown, with black deposit	Brown, with black deposit
Sodium metabisulphite 0·1 per cent	Deep pink	Deep brownish-pink	Purplish-red	Brown, with black deposit
Sodium metabisulphite 0·2 per cent	Deep pink	Red, with black specks	Purplish-red with black deposit	Brown, with black deopsit
Disodium edetate 0·1 per cent	Colourless	Colourless	Colourless	Colourless

results in Table I are for the half-filled 15 ml. amber-coloured dropper bottles. The values for the other samples under test followed a similar pattern, although sodium metabisulphite was more effective in preventing coloration in white bottles than in amber-coloured bottles. In amber bottles metabisulphite may even accelerate the coloration. Colourless solutions containing edetate showed no loss of activity when tested

TABLE II

THE EFFECT OF ADDING HYDROGEN PEROXIDE (100 VOL.) TO 10 PER CENT SOLUTION OF PHENYLEPHRINE IN THE PRESENCE OR THE ABSENCE OF PRESERVATIVES IN FULL WHITE GLASS MACARTNEY BOTTLES

Preservative added	Storage period (days)				
	0·2	1	3	10	90
None	Deep red	Black with copious deposit	Black with copious deposit	Black with copious deposit	Black with copious deposit
Sodium metabisulphite 0·1 per cent ..	Colourless	Colourless	Pink	Deep brown	Black with copious deposit
Sodium metabisulphite 0.2 per cent ..	Colourless	Colourless	Pink	Pale brown	Deep brown
Disodium edetate 0·1 per cent	Colourless	Colourless	Colourless	Colourless	Light orange

biologically and the brown solutions with black deposits had lost not more than 10 per cent of total activity.

Further solutions were then prepared and treated with hydrogen peroxide (0·2 ml. 100 vol./15 ml. solution) to accelerate the oxidation processes. The results shown in Table II indicated that disodium edetate exerted a strong protective action against the oxidative property of hydrogen peroxide. Sodium metabisulphite was less effective. The black and brown solutions again showed insignificant losses of activity.

These results confirm the observation that solutions of phenylephrine colour on oxidation but the amount of colour is no indication of the decrease in activity. Sodium metabisulphite delays the colour formation but disodium edetate (0·1 per cent) is better. The protective effect of edetate is generally believed to be due to its chelating power (on metals such as iron and manganese, two constituents of amber glass[3]) but it also exerts an antioxidant effect. Disodium edetate is harmless to the eyes and is therefore recommended as the preservative for solutions of phenylephrine (10 per cent).

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After Dr. Whittet presented the paper there was a DISCUSSION. The following point was made.

For the treatment of calcium burns in the eye an 11 per cent solution of disodium edetate was now being used without untoward effects.

Short Communication

PRELIMINARY STUDIES OF THE HEAT RESISTANCE OF BACTERIAL SPORES ON PAPER CARRIERS

By A. M. Cook and M. R. W. Brown

From the Department of Pharmaceutics, School of Pharmacy, University of London, Brunswick Square, London, W.C.1

Received May 23, 1960

Recently there has been a reappraisal of the methods of testing sterilisers and sterilising techniques[1–4] and spore impregnated papers have been introduced for this purpose. The spore papers* are available as paper strips, impregnated with spores of *Bacillus stearothermophilus*, sealed in glassine envelopes. Spores on these strips are stated to survive 5 minutes at 121° moist heat, but to be killed after 12 minutes.

The resistance to moist heat at 121° was tested in an autoclave, the lid of which contained a plunger-type device to facilitate the rapid movement of the spore papers in and out of the autoclave. The temperature in the autoclave was recorded by a thermometer and a pressure gauge.

TABLE I

Effect of recovery in medium, A, B or C on the apparent survival time of spore strips exposed to steam at 121°

Time in minutes at 121°	Recovery broth		
	A	B	C
3	+ – – – –	+ + + + +	+ + + + –
3½	+ – – – –	+ + + + +	+ + + + +
4	– – – – –	+ + + + +	+ + + + +
4½	– – – – –	+ + + + –	+ + + + –
5	– – – – –	+ + + – –	+ + + + +
5½	– – – – –	+ + + + –	+ + + – –
6	– – – – –	+ + – – –	+ + + + +
6½		+ – – – –	+ + + + –
7		– – – – –	+ – – – –
7½		+ – – – –	+ + + – –
8		+ – – – –	+ – – – –

Each symbol indicates the response of one spore strip.
+ = growth.
– = no growth.

The spore strips were cut into halves and several halves fitted separately on to the plunger barrel which was then brought into contact with steam at 121°. The time of contact was measured by a stop watch, and at the end of this the plunger was withdrawn from the steam. Thermocouple readings indicated that the spore strips reached the temperature of the steam in less than 8 seconds. Immediately after removal from the steam, the strips were transferred to the recovery medium, incubated at 55° and examined at intervals for up to 2 weeks.

Three kinds of recovery medium were used in 10 ml. quantities.

* Oxoid Spore Strips, Code number BR23, Oxoid Division, OXO Ltd., London, E.C.4.

(A) Oxoid Dextrose Tryptone Broth, Code number CM73 containing 0·5 per cent dextrose, 1 per cent tryptone (Oxoid), 0·004 per cent brom-cresol purple. This is recommended as a recovery medium by the makers of the spore strips.

(B) Broth containing 0·5 per cent dextrose, 1 per cent tryptone (Difco).

(C) Broth containing 0·5 per cent dextrose, 1 per cent tryptone (Oxoid).

The results of over 300 observations of the effect of recovery in medium A or B on the apparent survival time of spore strips after exposure are given in Figure 1. Experiments using medium C are as yet incomplete and the data have not been included in the Figure. However, sufficient

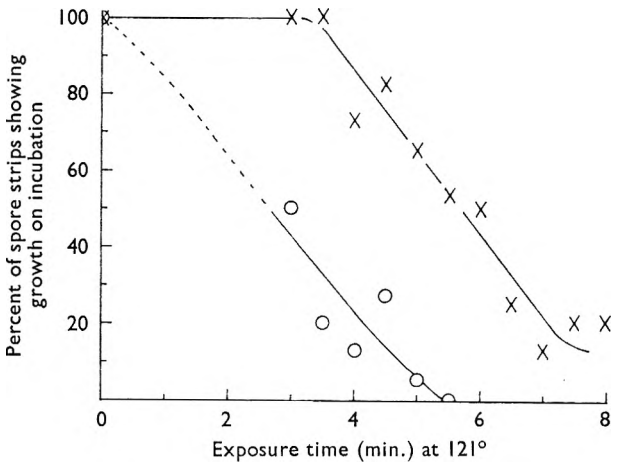

FIG. 1. Effect of recovery medium on the apparent survival time of spore strips exposed to steam at 121°.
○, recovery medium A; X, recovery medium B.

results have been obtained to justify preliminary comparison with results from A and B. Table I gives the combined results of two separate experiments using A, B and C as recovery media.

The claim made for the survival of these spores has not been substantiated by our results (Fig. 1). When using the recovery medium A, 50 per cent of the spore strips failed to show growth after 3 minutes' exposure.

These results also show that all the spore strips after 3 minutes' exposure were capable of producing growth when recovered in medium B but the percentage dropped rapidly with increased heating time.

Table I indicates that there is little, if any, difference between broths B and C in their ability to support growth from heated spore strips.

Since medium A is medium C with added brom-cresol-purple, it is likely that the absence of the dye from the recovery medium accounts for the increase in the apparent survival time of heated spore strips which occurs with medium C.

Most of the spore strips which showed growth on incubation in the recovery media did so within 24 hours. Growth was not observed after 14 days with any spore strip which had failed to show growth after 36 hours.

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After Mr. Brown presented the paper there was a DISCUSSION. The following points were made.

The introduction of the plunger did not influence the temperature: pressure relationship in the autoclave. The work had not set out to prove or disprove the manufacturer's claims for spore papers, but was part of a study on the heat resistance of spores. The organisms used were unlikely to withstand the conditions in dressing sterilisers working at 30 p.s.i. for 10 minutes.

MOULD SPORE SUSPENSIONS AND POWDERS FOR USE IN FUNGICIDAL KINETIC STUDIES

PART I. PRELIMINARY EXPERIMENTS WITH *Rhizopus nigricans* AND *Penicillium digitatum*

BY M. R. W. BROWN* AND KENNETH BULLOCK

From the Department of Pharmacy of the University of Manchester

Received May 17th, 1960

Reproducible colony counts of spore suspensions of *Rhizopus nigricans* and *Penicillium digitatum* have been obtained on a malt medium containing 0·02 per cent rose bengal. Spores of these organisms suspended in different strengths of Ringer's solution and stored at 1–4° lost viability after a lag of up to 2 days. A spray-dried peptone powder containing evenly distributed *R. nigricans* spores has been prepared without loss of viability of the spores.

INVESTIGATIONS previously made in this department related the effects of storage, moisture, heat, disinfectants and ionising radiations to the viability, as judged by roll-tube colony counts, of *Bacillus subtilis* spores in aqueous suspensions, powders and oils[1–4]. The object of this work was to ascertain how far these investigations could be extended to similar preparations of mould spores.

Many methods of evaluating antifungal agents by their action on mould spores have been described[5]. With one exception[6], these methods have not enabled the results to be statistically analysed and used in kinetic studies comparable with those employed in the study of bactericides.

EXPERIMENTAL METHODS

Choice of Test Organisms

Rhizopus nigricans and *Penicillium digitatum*, both previously recommended as test organisms[7,8], were chosen because the spores of these mould separate readily into single spores, are water-wettable, grow freely on common types of medium and are large enough for direct microscopic examination. *R. nigricans* was used as the main test organism and *P. digitatum* to check selected results.

Counting Method

The techniques of standardising apparatus, making roll-tube colony counts and assessment of errors were those previously described[9,10]. The mean coefficient of variation was 4·4 per cent for *R. nigricans* and 2·2 per cent for *P. digitatum*. The goodness of fit of the values of χ^2, obtained from 100 quintuplicate counts on suspensions of *R. nigricans* spores is recorded in Table I.

Preparation of Suspensions of Spores

Malt extract was chosen as the basic medium[11,12] because the growth of both test organisms is favoured by abundant carbohydrate and slight acidity. Water used in the media was glass-distilled.

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Stock cultures of the test organisms were grown on a medium which contained 3 per cent Malt Extract in water, solidified with 1·5 per cent agar (medium A) and incubated at 25° for 1 week after which they were stored at 3° for up to 3 months.

Preliminary experiments showed that growth of *R. nigricans* in a liquid malt medium stimulated abundant sporulation and consequently spore

TABLE I

GOODNESS OF FIT OF VALUES OF χ^2 OBTAINED FROM COUNTS OF SUSPENSIONS OF *R. nigricans* SPORES USING SETS OF FIVE ROLL-TUBES

Value of χ^2	Expected frequency (m)	Observed frequency	Difference (x)	$\frac{\chi^2}{m}$
Under 1	9·02	11	1·98	0·43
Between 1 and 2	17·40	23	5·60	1·89
Between 2 and 3	17·79	19	1·21	0·08
Between 3 and 4	15·18	14	1·18	0·09
Between 4 and 5	11·87	10	1·87	0·30
Between 5 and 6	8·82	7	1·82	0·38
Between 6 and 7	6·33	6	0·33	1·72
Between 7 and 9	7·48	5	2·48	0·83
Over 9	6·11	5	1·11	0·21

$\chi^2 = 5{\cdot}88$ N = 8 P = 0·5–0·7

TABLE II

DISTRIBUTION OF SPORE GROUPS IN SPORE SUSPENSIONS OF THE TEST ORGANISMS

Organism	Suspension	Approximate concentration of suspension in millions per ml.	Number of slides examined	Percentage of single spores
Rhizopus nigricans	A	10	27	92
	B	16	10	92
	K	10	17	90
	Dilution of K	3	25	97
	Q	14	10	93
	Dilution of Q	6	10	95
	Further dilution of Q	3	13	97
Penicillium digitatum	M	6	5	94
	N	2	5	93
	P	7	5	94

In suspensions of the test organisms containing less than 3 million spores per ml. none of the clumps contained more than 2 spores.

suspensions of this organism were prepared by growing on a medium, containing 3 per cent Malt Extract in water, for 14 days at 25°. The resultant mycelial felts, bearing sporangia, were removed from the surface of the medium and shaken with water to remove the spores. *P. digitatum* was grown on slopes of medium A for 14 days at 25° when the spores were washed off. With both test organisms the crude spore suspensions were strained through gauze and run from a burette so that the last portion

which contained floating debris could be discarded. The spores were collected by centrifuging and resuspended in water.

Spore suspensions of the test organisms were examined microscopically to determine the percentage of single spores and the results are recorded in Table II. Sixteen fields, each containing approximately from 5 to 12 spores depending upon the concentration of the suspension used were examined on each slide.

Discrepancy Between Total Count and Viable Count

During this work it was observed that the colony counts for both test organisms were only about half the total count obtained by calculating the mean of three counts using Thoma chambers.

Investigation of the Inhibitory Effect of Rose Bengal on the Growth of the Test Organisms

The excessive spreading of fungal colonies was a serious difficulty and prevented an accurate colony count. To reduce their size it has been recommended[6] that the medium should be more concentrated than that

TABLE III

MAXIMUM PERCENTAGE GERMINATION OF *Rhizopus nigricans* SPORES

Experiment	Number of plates examined	Number of fields per plate	Maximum percentage germination	
			Standard medium	Rose bengal medium
A	3	10	89	90
B	3	10	90	94
C	3	10	93	93

used for normal culture. This procedure alone was found unsatisfactory and experiments were made to investigate the effects of rose bengal on the growth of the test organisms since this dye in a concentration of 1 in15,000 has been recommended[13] as an anti-spreading agent.

Microscopic determination of the maximum percentage germination. Petri dishes containing medium A as a standard, and dishes containing medium A with 1 in 15,000 rose bengal added were streaked with a suitable dilution of a spore suspension of *R. nigricans* and incubated at 25°. The spores were examined microscopically and the maximum number of spores which germinated is recorded as a percentage in Table III. Each field viewed contained about 20 spores.

Effect of rose bengal on the colony count. A roll-tube medium which contained 6 per cent Malt Extract solidified with 2·5 per cent agar was used as a standard and was compared with a medium containing the same constituents with 1 in 15,000 rose bengal. Five roll-tubes of each medium were inoculated with 1 ml. quantities from the same dilution of a spore suspension of *R. nigricans*. The dilutions were adjusted to give counts of not more than about 50 colonies per roll-tube to prevent overlap of the colonies that would otherwise occur on the standard medium. The results were compared by means of the *t* test and are seen in Table IV.

Experiments with *R. nigricans* have indicated that concentrations of rose bengal of up to 1 in 3,000 in the standard medium do not reduce the colony counts when compared with those obtained by using 1 in 15,000 rose bengal[10]. But as the concentration of rose bengal increased it became

TABLE IV

RESULTS OF COMPARISONS OF COLONY COUNTS OF THE TEST ORGANISMS OBTAINED ON MEDIA CONTAINING DIFFERENT CONCENTRATIONS OF ROSE BENGAL

Media compared by *t* test	Organism	Experiment (replicates)	*t*	P
(*a*) 6 per cent Malt Extract	*Rhizopus nigricans*	A	0·8974	0·4–0·5
(*b*) 6 per cent Malt Extract and 1 in 15,000 rose bengal		B	0·6822	0·5–0·6
		C	0·0623	0·9
a) 6 per cent Malt Extract and 1 in 15,000 rose bengal	*Rhizopus nigricans*	D	0·3556	0·7–0·8
(*b*) 6 per cent Malt Extract and 1 in 5,000 rose bengal		E	0·2192	0·8–0·9
		F	0·8082	0·4–0·5
(*a*) 6 per cent Malt Extract	*Penicillium digitatum*	M	0·4150	0·6–0·7
(*b*) 6 per cent Malt Extract and 1 in 5,000 rose bengal		N	0·7960	0·4–0·5
		O	0·8781	0·4–0·5

easier to count large numbers of colonies. Consequently, the experiment was repeated using the standard medium containing 1 in 5,000 and 1 in 15,000 rose bengal and the colony counts obtained were compared (Table IV).

Colony counts of *P. digitatum* spores on the standard medium with 1 in 5,000 rose bengal added were compared with those obtained without the dye (Table IV).

The reproducibility of the medium containing 1 in 5,000 rose bengal and 6 per cent Malt Extract was tested by inoculating 5 roll-tubes from

TABLE V

RESULTS OF COMPARISONS OF COLONY COUNTS OBTAINED ON SUCCESSIVE BATCHES OF MEDIA

Batches compared by *t* test	*t*	P
1 and 2	0·4999	0·6–0·7
2 and 3	0·6163	0·5–0·6
3 and 4	1·0236	0·3–0·4

successive batches with 1 ml. taken from the same dilution of a suspension of *R. nigricans* spores. The counts obtained from successive pairs of batches were compared by means of the *t* test (Table V).

Viability of spore suspensions after storage. To test for any loss of viability of the spores on storage, suspensions of the test organisms were stored at 3° and colony counts were made after the time intervals shown in Figure 1. In this experiment the spores were suspended in water and in dilutions of Ringer's solution.

Spray drying of suspensions of spores. The apparatus and techniques of the spray drying process have been previously described[14]. A suspension of *R. nigricans* spores was added to a 10 per cent solution of peptone in water, previously cooled to about 3°, so as to give a colony count of about 20,000 per ml. This suspension was cooled by an ice-water jacket

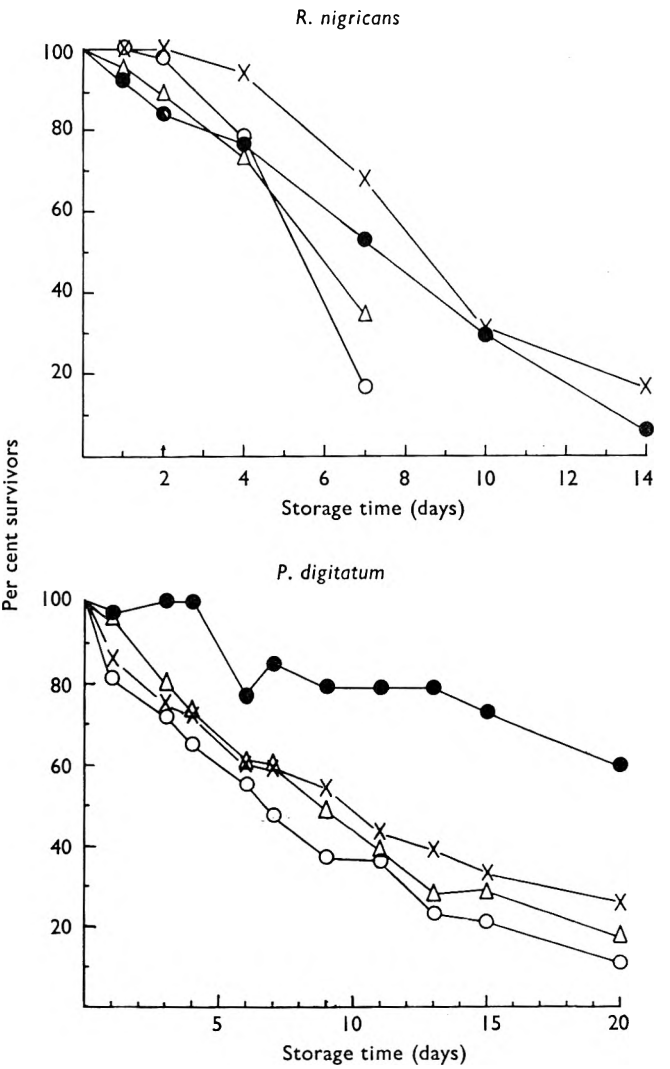

FIG. 1. Effect of storage at 3° upon the colony count of spores of the test organisms suspended in water and in different strengths of Ringer's solution.

● Water.
○ Ringer's solution.
△ ½ strength Ringer's solution.
× ¼ strength Ringer's solution.

and fed to the spray drier with a main inlet temperature of 80°. The spray dried powder was mixed in a revolving jar for 24 hours. Evenness of distribution of the spores was confirmed by an analysis of variance of the viable counts of 8 random samples of the powder each made in quintuplicate. See Tables VI and VII. Colony counts of the reconstituted

TABLE VI

QUINTUPLICATE PLATINGS OF 8 SAMPLES OF THE SAME SPRAY-DRIED POWDER CONTAINING *R. nigricans* SPORES

Sample	1	2	3	4	5	6	7	8
Weight of powder (g.)	0·0680	0·0675	0·0501	0·0664	0·0528	0·0679	0·0589	0·0712
Volume of diluent (ml.)	6·8	6·8	5·0	6·6	5·3	6·8	5·9	7·1
Roll-tube counts	194	171	210	193	184	200	198	190
	189	180	180	177	183	183	200	185
	210	198	183	172	174	189	190	196
	187	202	210	211	188	195	204	192
	194	207	200	201	182	176	197	200
Total	974	958	983	954	911	943	989	963

TABLE VII

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATINGS OF 8 SAMPLES OF THE SAME SPRAY-DRIED POWDER

Source of variation	Sum of squares	N	Mean square	Variance ratio	P
Difference between samples	860·4	7	122·9	1·02	0·2
Difference between individuals	3866·0	32	120·8	—	—
Total	4726·4	39	—	—	—

TABLE VIII

COLONY COUNTS OF *R. nigricans* SPORES IN A SPRAY-DRIED PEPTONE POWDER AFTER DIFFERENT PERIODS OF STORAGE

Period of storage (days)	0	12	24	35	53
Count (mean count of 5 roll tubes)	173	196	198	166	173

suspension showed no loss of viability of the spores as a result of the spray drying process. Colony counts of the powder were made after the time intervals as shown in Table VIII.

DISCUSSION

It has been shown (Table II) that suspensions of the test organisms can be prepared containing about 97 per cent of single spores for *R. nigricans* and about 94 per cent for *P. digitatum.*

Figure 1 shows that spores of the test organisms rapidly lost viability when suspended in water and in dilutions of Ringer's solution. The suspensions were examined microscopically for the production of germ tubes and none were observed. However, germination might have advanced to a stage which preceded germ tube production but at which

viability was rapidly lost due to lack of nutrients. Consequently it was decided to use freshly prepared spore suspensions of the test organisms. *R. nigricans* spores were suspended in one-quarter strength Ringer's solution and *P. digitatum* in water since under these conditions there was a lag of about 2 days before viability decreased.

The mean coefficients of variation for both test organisms are regarded as satisfactory and may be compared with 3·79 per cent obtained by Berry and Michaels[15] who have designated this figure as the standard error of the counting method since it represents the sum of the variances due to manipulative technique, sampling and counting errors, and errors due to variability of medium. The goodness of fit of values of χ^2 shown in Table I is satisfactory and indicates that the counting technique is capable of giving reproducible results.

Table III shows that both the standard medium and the rose bengal medium allow about 90 per cent germination of suspensions of *R. nigricans* spores.

The presence of 1 in 5,000 rose bengal in the roll-tube medium did not lower the colony count of spore suspensions of either test organism when compared with that obtained on the standard malt medium (Table IV), but it facilitated the counting of the colonies. On the basis of these results the roll-tube medium chosen for subsequent work was Malt Extract 6 per cent, rose bengal 1 in 5,000, agar 2·5 per cent, water to 100 per cent. Table V shows that different batches of this medium offer similar facilities for the growth of colonies.

There is an apparent conflict between the results obtained in this work with rose bengal and those obtained by Smith and Dawson[13]. They found that a concentration of rose bengal of 1 in 10,000 reduced the colony count of soil fungi while a medium containing smaller amounts of rose bengal did not do so. However, these workers did not use a known suspension of single spores of one species but used a soil suspension as a source of mixed organisms. It is possible that the rose bengal increased the lag phase of growth by an amount which differed for various fungal species so that fast growing fungi may have produced colonies which inhibited the growth of more slowly developing fungi. Bain[16] has since confirmed our findings with rose bengal and using a similar technique to that we have described has obtained reproducible colony counts of spore suspensions of *Penicillium spinulosum* and *Aspergillus niger*.

Table VIII shows that colony counts of *R. nigricans* spores in a spray-dried peptone powder did not decrease significantly during 7 weeks. It is intended that similar preparations of free flowing, spray-dried powders containing evenly distributed mould spores be used to study the viability of moulds in systems of low moisture content.

Experimental results have indicated that whilst about 90 per cent of *R. nigricans* spores are capable of developing germ tubes on the surface of agar plates (Table III), only about 50 per cent are capable of producing colonies in roll-tubes, and similar results have been obtained with *P. digitatum*[10]. For suspensions of bacterial spores it has been found possible[17,18] to reduce the discrepancy between the total and colony

counts of some organisms by heat and chemical stimulation of the spores. As yet such treatment has not been attempted. However, the cause of this discrepancy should be investigated before these test organisms are used in fungicidal experiments designed to correlate uptake of fungicide and rate of kill.

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MOULD SPORE SUSPENSIONS AND POWDERS FOR USE IN FUNGICIDAL KINETIC STUDIES

PART II. PREPARATIONS USING *Penicillium spinulosum*

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Received May 2, 1960

Spore suspensions of *Penicillium spinulosum* have been prepared containing over 99·0 per cent single spores in an even distribution. About 80 per cent of the spores were able both to produce germ tubes and to form colonies in a roll-tube. A peptone powder containing an even distribution of spores has been prepared. The viable count of the powder did not show a significant fall during 5 months storage. The suspension has been successfully used to study the sporicidal and fungistatic activities of chlorocresol solutions.

BROWN and Bullock[1] found that 80 to 90 per cent of *Rhisopus nigricans* and *Penicillium digitatum* spores put out germination tubes in the custom-moist slide germination test, yet roll-tube counts of suspensions of these spores were approximately half the corresponding total (haemocytometer) counts. This failure to produce colonies in a roll-tube might be a general characteristic of mould spores or it might be peculiar to the two species used by Brown and Bullock[1]. Bain[2] working in the same laboratory and using Brown's[3] counting technique found some evidence that the same difficulty might not arise with the spores of *P. spinulosum*. The present work was undertaken to investigate more fully the suitability of *P. spinulosum* spores for use in this type of work and, if possible, to use a suspension of these spores to evaluate the antifungal activity of Chlorocresol B.P.

EXPERIMENTAL AND RESULTS

Much of the work has been duplicated by two of the authors H.N.G.[4] and A.V.H.[5]. Where conditions, techniques or results differed, those of the former will be denoted by (G) and those of the latter by (H).

Penicillium spinulosum strain 42237 of the Commonwealth Mycological Institute was used. Stock cultures were incubated on slopes containing 3 per cent Liquid Malt Extract, and 2 per cent agar, at 25° for 10 weeks (G) or 21 days (H) and subsequently stored at 4°. Spore suspensions were prepared in two ways.

(G) Preliminary experiments showed that mature spores, present on 10 day old but more abundant on 21 day old cultures, are readily detached from the conidiophores and from one another. Suspensions were therefore prepared by inoculating of the same medium from the stock culture and incubating for 21 days at 25°. The mature spores were washed off the surface with distilled water and wetted by shaking vigorously in a test-tube. After passing through a sintered glass filter SG 3 the resulting suspension consisted of 99·4 per cent single spores and none of the clumps contained more than 2 spores.

(H) To minimise the effect of a possible mutant, malt agar slopes were heavily inoculated with a mixture of spores and hypae and incubated for 17 days at 25° after which the surface of the culture was flooded with sterile water. The spore suspension was withdrawn and "atomised" by

TABLE I

THE RESULT OF STATISTICAL ANALYSES ESTABLISHING THE SUITABILITY OF THE MEDIUM USED AND ASSESSING THE ERRORS INVOLVED IN ROLL-TUBE VIABLE COUNTS OF SUSPENSIONS OF THE SPORES OF *Pencillium spinulosum*

	(G)	(H)
Reproducibility of medium *t* test, 3 pairs of 4 batches	P 0·2–0·1 0·5–0·4 0·8–0·7	P 0·8–0·9 0·7–0·8 0·6–0·7
Sensitivity of the medium obtained from the goodness of fit of χ^2 from replicate viable counts	20 counts N = 19 χ^2 = 10·94 P = 0·95–0·90	18 counts N = 17 χ^2 = 13·6 P = 0·5–0·7
Overall errors of diluting and pipetting mean coefficient of variation	20 quintuplicate counts 4·83 per cent	10 quintuplicate counts 1·87 per cent
Normal sampling variance. Goodness of fit of χ^2	100 quintuplicate counts N = 8 χ^2 = 6·163 P = 0·5–0·7	60 quintuplicate counts N = 8 χ^2 = 7·97 P = 0·3–0·5

passing 3 times through a hand spray. The resultant suspension contained 85 per cent of single spores and 15 per cent of clumps of two spores.

Statistical analyses of a large number of roll-tube colony counts of both types of suspension of *P. spinulosum* spores using the technique and rose bengal medium described by Brown[3] are summarised in Table I. Limits of error are similar to those previously reported using *Bacillus subtilis*, *R. nigricans* and *P. digitatum*[3,7].

TABLE II

COMPARISON OF TOTAL HAEMOCYTOMETER COUNTS AND VIABLE ROLL-TUBE COUNTS IN MILLIONS OF SPORES PER ML.

Experiment number	1	2	3	4	5	6	7	8	9	Mean
Total count	6·7	4·75	6·75	8·5	7·15	7·15	7·1	6·0	5·1	
Method A										
Viable count	5·79	3·79	5·25	5·32	5·85	6·08	5·13	5·28	4·35	
Per cent viability	86·4	79·7	77·8	62·6	81·8	85·0	72·2	88·0	85·3	79·86
Method B										
Viable count	5·87	4·18	5·64	6·98	7·01	6·88	6·10	6·26	5·24	
Per cent viability	87·6	88·1	83·6	82·1	98·0	96·2	85·9	104·3	102·7	92·0

Relation between Total Count, Viable Count and Percentage Germination

(G) Total and colony counts were made on 9 different spore suspensions containing about 7×10^6 spores per ml. Total counts were made on a Thoma slide (Table II). Preliminary experiments had shown that when a spore suspension is measured using a dry pipette, the water retained in the pipette contained a lower concentration of spores than did the original suspension. Making conventional serial dilutions effects a concentration

of spores greater than would be expected in the higher dilutions. Two methods of dilution were therefore used. This concentration effect with *P. spinulosum* spores has been investigated and will be reported elsewhere.[8]

(H) Drops of a suspension containing about 10^6 spores per ml. in sterile tap water on a microscope slide were incubated at 25° for 24 and 48 hours in a moist chamber (closed Petri dish containing some moist filter paper). 300 spores on each slide were examined for formation of a germ tube. Table III shows that approximately 80 per cent of the mature spores germinated, a figure in close agreement with that for the percentage forming colonies.

Spores on the surface of malt agar, which after 24 hours at 25° were observed microscopically to have formed germ tubes, were transferred to a

TABLE III

DETERMINATION OF THE PERCENTAGE GERMINATION OF *P. spinulosum* SPORES IN SUSPENSIONS

Age of the culture from which the spore suspension was prepared (days)	Percentage germination of spores (mean of four slides)	
	After 24 hours incubation	After 48 hours incubation
4	1	1
5	5	19
6	2	—
14	24	50
15	46	52
16	60	71
17	79	80
18	72	79
21	—	82
22	62	77
30	87	87

second plate of malt agar, covered with thin layer of the same medium and incubated again at 25°. Out of 165 spores with germ tubes, 161 produced colonies in 24–48 hours, i.e., 97·5 per cent.

Preparation of a Powder Containing an even Distribution of P. spinulosum *Spores*

Using the method and conditions described by Brown and Bullock[1] a suspension containing about 2×10^5 *P. spinulosum* spores per ml. in 10 per cent peptone solution was spray dried. About 3 per cent mortality was found on drying. This was within the experimental error of counting. All the 13 viable counts obtained during a period of 5 months, when examined statistically, were the same within the error of counting showing that a stable powder had been produced. An analysis of variance (Tables IV and V) showed that the spores were evenly distributed in the powder.

Sporicidal Effects of Chlorocresol

A suspension containing about 10^5 spores in 10 ml. was mixed (time of running in 20 sec.) with 10 ml. of chlorocresol solution of twice the concentration to be investigated. In these preliminary experiments this reaction mixture was kept at room temperature. At the desired intervals

1 ml. quantities of reaction mixture were diluted (20 sec.) by addition to 99 ml. of sterile water. Six separate 1 ml. quantities of the well mixed dilution were transferred to roll-tubes and counted after 48 hours incubation at 24°. To check that this dilution was sufficient to quench the sporicidal activity of the chlorocresol, colony counts of *P. spinulosum* in

TABLE IV

REPLICATE VIABLE COUNTS OF 10 RANDOM SAMPLES OF SPRAY-DRIED PEPTONE POWDER CONTAINING *P. spinulosum* SPORES

Sample No.	Count per g. × 10^{-4}	Sample No.	Count per g. × 10^{-4}
1	132	6	136
2	134	7	102
3	113	8	97
4	123	9	107
5	118	10	137

roll-tubes containing a concentration of 0·001 per cent of chlorocresol in the medium were made and no reduction in numbers were observed. A control experiment with sterile water in place of chlorocresol solution was always run to ascertain the correct figure for the initial count. Timing was from the last drop of chlorocresol added to the spores to the diluting out of the reaction mixture. It was estimated that, because of the delivery time from the burette and pipette, some spores might have been exposed

TABLE V

THE ANALYSIS OF VARIANCE OF THE VIABLE COUNTS OF 10 SAMPLES OF SPRAY-DRIED POWDER

Source of variations	Sum of squares	N	Mean squares
Difference between samples ..	2,037·8	9	226·4
Difference between individuals	7,080·4	20	354·0
		29	

Variance ratio = 1·563
P = 0·2

to a varying concentration of chlorocresol for up to 28 seconds longer than others. This mixing-time factor obviously had the greatest effect in the experiment using 0·1 per cent chlorocresol, where 90 per cent of the spores failed to produce colonies after 2 minutes exposure. In this experiment the reaction mixture was kept in a 10 ml. burette so that 1 ml. quantities could be run off at 5 second intervals. Figure 1 shows the time against log per cent survivor curves using 0·05, 0·066 and 0·1 per cent chlorocresol solutions.

Fungistatic Experiments with Chlorocresol

In these experiments it was necessary to have accurately known and increasing concentration of chlorocresol in the roll-tube medium. Four ml. quantities of a medium containing 7·5 per cent malt extract, 0·025 per cent rose bengal and 3·75 per cent agar were delivered from a heated

container by means of a Matburn pressure operated ampoule filling apparatus into the roll-tubes. The mean delivery weight was 4·1190 g. (coefficient of variation 0·07 per cent. After autoclaving the plugged tubes the mean weight was 4·1177 (coefficient of variation 0·75 per cent.). In the experiments 1 ml. of chlorocresol solution 6 times the concentration

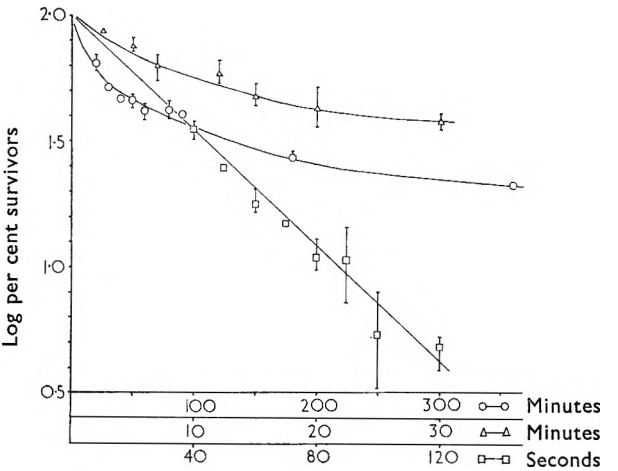

FIG. 1. The sporicidal effects of chlorocresol on spores of *P. spinulosum* in aqueous suspension at room temperature. Three experiments were made with each strength of chlorocresol. Experimental points denote the average values; vertical lines indicate variation. ○—○ = 0·05 per cent, △—△ = 0·066 per cent, □—□ = 0·1 per cent chlorocresol.

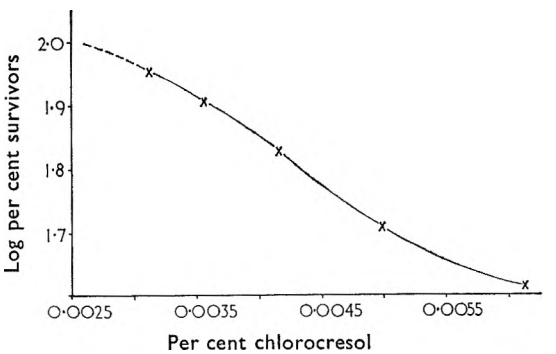

FIG. 2. Fungistatic effects of chlorocresol on the spores of *P. spinulosum* at 25°.

which it was desired to test and 1 ml. of a spore suspension having a viable count of 70 per ml. was added to each tube. In the critical strengths the chlorocresol not only prevented some of the spores from producing colonies, but also reduced the rate of colony growth. The control tubes containing no chlorocresol attained their maximum growth after 48 hours at 24°. All tubes containing chlorocresol could be counted after five days incubation. The count in those tubes containing chlorocresol 1 in

16,000 and 1 in 20,000 continued to increase up to 8 days incubation, whereas those containing chlorocresol 1 in 24,000 and 1 in 28,000 gave the same count as recorded after 5 days incubation. Tubes containing chlorocresol 1 : 32,000 showed confluent growth after 8 days incubation. In these experiments therefore the counts were recorded after 48 hours for the controls, after 5 days with 1 in 32,000 and after 8 days with all the other strengths of chlorocresol. The results are shown in Figure 2.

Discussion

From the work described and from the earlier work of Berry and Perkins[6] and Brown and Bullock[1] it appears that aqueous suspensions of the spores of different species of moulds can be prepared which are suitable for studying the kinetics of fungicidal action. As a test organism *P. spinulosum* offers certain advantages. The spores are of a medium size but large enough for microscopic observation of germ tubes. On rose bengal medium they form discrete compact easily counted colonies. As shown in Tables II and III there is a close agreement between the percentage of the spores forming colonies in a roll-tube and the percentage putting out a germ tube in a moist chamber. Further, as Table II shows, the viable count is about 80 per cent of the total count which is better than the 50 per cent found by Brown and Bullock for *R. nigricans* and *P. digitatum*.

The best way of obtaining a spore suspension containing the highest proportion, over 99 per cent, of single spores is method G described above using a 21 day old culture and filtration through a sintered glass filter SG 3.

Table I confirms that colony counts of mould spore suspensions can be replicated with errors comparable to those found with colony counts of *B. subtilis* spore suspensions. It should be pointed out that favourable results of statistical analyses of viable counts can be obtained with spore suspensions which are not ideal since they were obtained by (*a*) Brown when only 50 per cent of the total spores were forming colonies and (*b*) by Brown and Harkiss above with suspensions containing different proportions of single and grouped (double) spores, as well as by Gerrard above with suspensions containing over 99 per cent single spores.

It has been found possible to prepare peptone powders stable on storage containing an even distribution of *P. spinulosum* spores. These are being used to investigate the effects of heat and gaseous fungicides on contaminated powders.

In experiments using 3 strengths of chlorocresol reasonably close agreement has been obtained between three replicate experiments with different spore suspensions. Except in the instance of 0·1 per cent chlorocresol where the experiment was completed in $2\frac{1}{2}$ minutes, the time log per cent survivor graphs appear not to be straight lines. The reason for the deviations from linearity needs investigation.

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7. Rawlins, Keepe and Bullock, *J. Pharm. Pharmacol.*, 1949, **1**, 878.
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After Mr. Brown presented Part I and Mr. Gerrard presented Part II there was a DISCUSSION. The following points were made.

The rose bengal medium had been successfully used for counting other organisms; a fungistatic effect had not been observed with high concentrations of the dye but the dye might enhance the fungistatic action of added substances. The toxic effects of Ringer's solution as a diluent were confirmed. Fungistatic concentrations should be clearly defined, as the concentration inhibiting the growth of the organism may not be the same as the concentration inhibiting germination of the spores.

Short Communication

THE EFFECT OF PIPETTING ON THE CONCENTRATION OF HOMOGENEOUS SPORE SUSPENSIONS

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Received April 8, 1960

Viable counts of micro-organisms are commonly used in the assessment of antibacterial substances. These usually involve microscopic counts of a suspension of test organisms followed by dilution before enumeration by roll tube methods.

Dilutions of about 10^{-3} are made by pipetting and it is assumed that these are accurate. We have found that certain unsuspected errors can occur in the preparation of diluted suspensions of micro-organisms and the factors involved have been investigated.

Spore suspensions of *Penicillium spinulosum* of various concentrations were prepared and the optical densities were found to be related to haemocytometer counts. Aqueous dilutions of spore suspensions, ranging from 1 in 4 to 1 in 10, were prepared using a calibrated 1 ml. pipette and a graduated 10 ml. burette. The optical densities of the volumetric dilutions were higher than the expected values, calculated from the dilution ratios. When the suspensions were diluted by weighing, these discrepancies did not occur. It was concluded that pipetting had a concentrating effect on the diluted suspension.

A volume of a spore suspension was withdrawn with a pasteur pipette from a test tube and ejected back. The optical density of the suspension before and after 24 such operations was found to be unchanged using the same pipette. When a fresh dry pipette was used for each operation the optical density of the suspension was found to increase by approximately 4 per cent.

Twenty-four serial transferences of suspension were made from test tube to test tube using a fresh pasteur pipette for each tube. Every six transferences increased the optical density by approximately 8 per cent.

A spore suspension drawn up a vertical capillary tube was allowed to descend and viewed through a microscope. Streamline flow of spores was observed. Spores at the periphery moved at a slower rate than those in the centre. The downward movement of peripheral spores changed as the meniscus "overtook" them, and they passed rapidly across under the meniscus and then joined the fast moving central stream. Spores were not left in the residual film on the capillary wall.

A glass tube (length 80 cm. bore 5 mm.), provided with a tap at the lower end, was filled with water. A concentrated spore suspension was layered both on the top and half way down the column. The liquid was allowed to run down the tube and the behaviour of the spore zones closely observed. The suspension layers assumed the form of progressively elongating parabolas and a clear layer appeared over the top of the upper

spore zone. The velocity at the tips of the parabolas was double that of the meniscus while the spores at the periphery of the lower suspension did not move. These results showed the radial velocity distribution of the spores to be in accordance with the Poiseuille equation, and indicated the return of slower peripheral spores to the faster moving central stream on approach of the meniscus, which travelled at half the speed of the fastest spore.

2 ml. of a spore suspension was drawn into a pipette and 1·0 ml. ejected into each of two test tubes. The optical densities of the original suspension and of the deliveries were 0·537, 0·538, and 0·548 respectively. Similarly deliveries of 1·5 and 0·5 ml. were made giving readings of 0·535, 0·542 and 0·554 (means each of eight experiments).

The spore concentration was higher in the last portion delivered, and was greater in the last quarter than in the last half. Since the fastest spores moved at twice the speed of the meniscus the first ml. was the same as the original. When deliveries of 1·5 and 0·5 ml. were made the 1·5 ml. delivery was composed of 1 ml. original strength and 0·5 ml. of concentrated suspension. The theoretical value would be

$$\frac{(2 \times 0{\cdot}535) + 0{\cdot}554}{3} = 0{\cdot}541$$

and the experimental value was 0·542.

Deliveries made between two graduation marks on a pipette, where the distance between the marks was less than that from the tip of the pipette to the lower mark, showed no increase in optical density.

Dilutions of 10^{-5} were made by (i) using two 1 ml. pipettes and dilutions of 100 and 1,000 times and (ii) by using five 1 ml. pipettes and five 10 times dilutions. Colony counts were made on roll tubes inoculated with one ml. samples of the last dilution. The colony count/total count ratio by the first method was 80 per cent, and 90 per cent by the second method.

It is usually assumed when spore suspensions are pipetted that the residual liquid remaining on the wall of the pipette has the same composition as the original suspension. This has not been found to be true; this can be a source of error in making serial dilutions. With *Penicillium spinulosum* spores the residual film contains few spores and pipetting has a concentrating effect. Similar effects have been observed with red blood cells suspended in saline and also by Copley[1]. The error may not occur with all organisms and may vary with techniques and workers. It can be allowed for by standardising techniques and be minimised by reducing the number of pipetting operations. Preferably the error can be eliminated by using a pipette with two graduation marks the distance between them being less than the distance between the lower graduation mark and the tip of the pipette. With dropping pipettes the drops should be delivered from a full pipette.

Acknowledgements. The authors are indebted to Dr. Maude of the University of Wales, Aberystwyth, for kind advice on hydrodynamical problems, and to Professor K. Bullock for permission to publish data from the M.Sc. Thesis of H. N. Gerrard.

REFERENCE

1. Copley, *Transactions of the Faraday Society*, September, 1959.

After Mr. Porter presented the paper there was a DISCUSSION. The following points were made.

B. subtilis spores had shown similar behaviour to that described for *P. spinulosum*. A thoroughly wetted tube eliminated the error with the mould spores.

NEUROMUSCULAR BLOCKING AGENTS

‡Part VII. Linear Polyonium Ethers

By D. Edwards*, J. J. Lewis†, D. E. McPhail*

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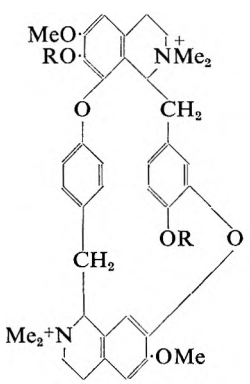
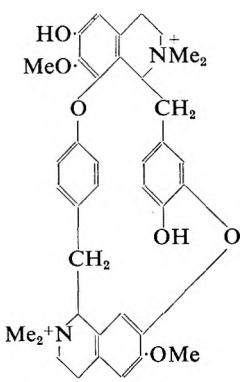
Received May 23, 1960

The tris-onium ethers (XIII, A and B) and the tetra-onium ethers (XV A, B and C), together with the corresponding polymethylene tris-onium (XIV A and B) and polymethylene tetra-onium compounds (XVI, A, B and C), have been synthesised. Replacement of a methylene group in the inter-onium polymethylene chain by an ether link almost always lowers muscle relaxant potency.

Before the introduction of muscle relaxants, ether was often the volatile anaesthetic of choice, since it is safe and gives good muscle relaxation. Ether not only possesses muscle-relaxant properties of its own[1–9], but it has also been shown to potentiate the actions of tubocurarine, gallamine and other non-depolarising relaxants. Many natural and synthetic non-depolarising muscle relaxants including tubocurarine, gallamine laudexium, prodeconium and oxydipentonium are themselves ethers, and, their potency is often dependent on the nature and position of the ether links[10]. Thus tubocurarine dimethylether (I, R = Me) is more potent than tubocurarine, (I, R = H)[10,11], though higher alkyl ethers of tubocurarine are less potent than the parent compound. The isomeric (+)-chondocurarine (II), which differs from (+)-tubocurarine solely in the position of the methoxyl and hydroxyl substituents, is approximately 2·9 times more potent than the latter in the rabbit[10]. Collier and



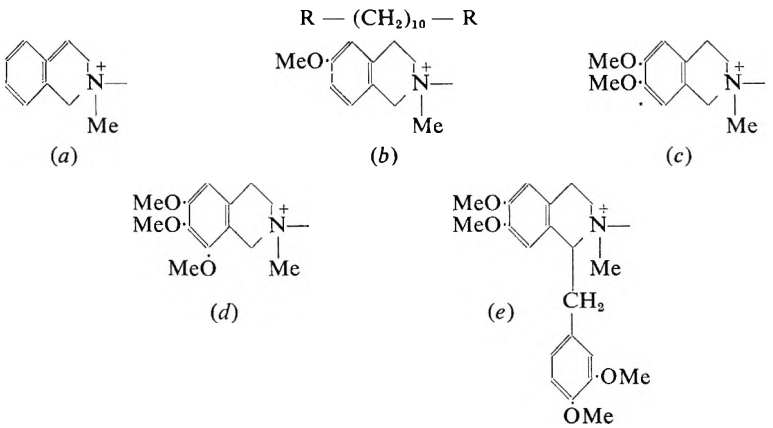
(I)



(II)

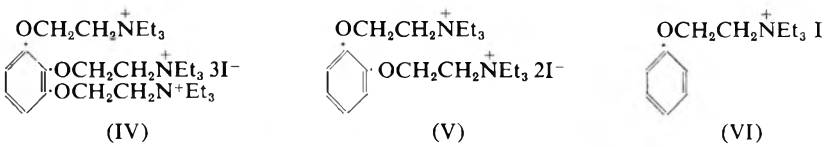
‡Part VI, see Reference 26.

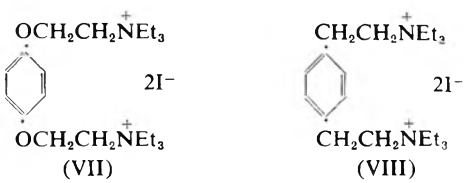
others[12,13] have shown similarly in a series of methylene bis-tetrahydroisoquinolinium (III*a*-III*d*) and related compounds that activity increases with aromatic methoxyl substitution. Laudexium (III*e*)[12–14] is one of the most potent curarising agents in this series.



(III)

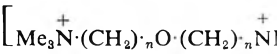
Bovet and others have synthesised and tested a large number of muscle relaxants containing ether links, including a series of ethers of choline and homologous amino-alcohols with mono- di- or tri-hydric phenols. Comparison of gallamine (IV), the most important compound of this group, with compounds 2559F (V) and 3697RP (VI) shows that potency increases as the number of β-(triethylammonium)-ethoxy groups increases[15]. Bovet and his colleagues[16] have shown, however, that compounds (VII) and (VIII) which are analogous, though not strictly comparable, are equipotent in the rabbit, and this suggests that potency is more a function of the number of ethonium groups rather than of the number of ether links.





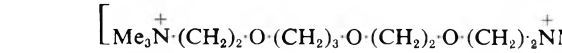
Aliphatic ethers with curarising activity are also known. In 1953 Levis and her colleagues[17,18] investigated a group of compounds which resembled the methonium compounds of Barlow and Ing[19] and Paton and Zaimis,[20] but containing one, two, or three ether oxygens in the interonium

chain. The compounds (IX, $n = 4$ or 5) were less potent than decamethonium, whilst a second or third oxygen in compounds (X) and (XI) progressively lowered potency. The view has been put forward by Girod and Häfliger[21] that the curarising potency in aliphatic compounds with two or more ether oxygens is related primarily to the number of methylene groups separating the ether oxygens, and their observations on the series of compounds (XII) suggest that this may be so. Thus activity

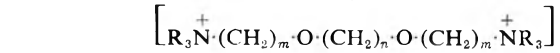


(IX)

(X)



(XI)

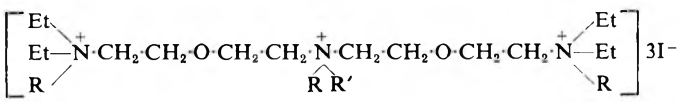


(XII)

falls progressively in the three compounds (XII) where R = Et, $n = 10$, $m = 2$; R = Et, $n = 8$, $m = 3$; and R = Et, $n = 6$, $m = 4$. This, however, leaves undetermined the question of the relative importance of inter-oxygen or oxygen-nitrogen distances, since further comparisons are between compounds of varying inter-onium spacing. Nor is this point clarified by the work of Hazard and Cheymol[29] and of Vanecek and Protiva[23] on similar compounds. The difficulties of assessing clearly the influence of ether oxygen upon curarising activity in aliphatic compounds are accentuated by the fact that different workers have used different preparations and species and that results for comparable non-ethers are not always available.

Chemical

The preparation of the tris-onium compounds (XIII, R′ = Et, R = Me and Et) and of the tetra-onium compounds (XV, R = Me and Et) incorporating the diethyl ether link is now described. The related polymethylene compounds (XIV, R = Me, Et and Pr) and (XVI, R = Me, Et and Pr) have also been prepared for comparison with the ether-linked derivatives.



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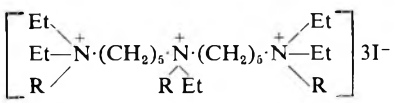

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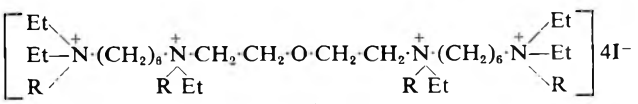
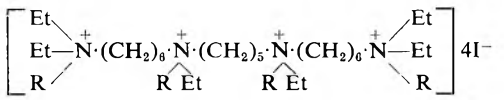


(XV)



(XVI)

The closely related compound (XIII, R = Et, R′ = Me) prepared by Protiva and Pliml[25] was obtained by condensation of diethylaminoethyl chloride with *N*-methyldiethanolamine in the presence of sodamide, and quaternisation of the resulting bis(2-diethylaminoethoxyethyl) methylamine. Although the latter was obtained in 57 per cent yield, the ready availability of diglycollic acid led to the choice of the following methods based on those used in our earlier work[25,26], for the preparation of bis(2-diethylaminoethoxyethyl) ethylamine.

Diglycollic anhydride prepared by dehydration of diglycollic acid with acetic anhydride[27], was treated with methanol to yield methyl hydrogen diglycollate[28], and the latter converted via the acid chloride to methyl *NN*-diethyldiglycollate (XVII). Reduction of the latter with lithium aluminium hydride gave an excellent yield of diethylaminoethoxyethanol (XVIII) which, however, was converted via diethylaminoethoxyethyl bromide hydrobromide into *N*(2-diethylaminoethoxyethyl)ethylamine (XIX) only in rather poor yield. Bromination of diethylaminoethoxy-

$Et_2N{\cdot}CO{\cdot}CH_2O{\cdot}CH_2COO{\cdot}Me$ (XVII)

$Et_2N{\cdot}(CH_2)_2{\cdot}O{\cdot}(CH_2)_2{\cdot}OH$ (XVIII)

$Et_2N{\cdot}(CH_2)_2{\cdot}O(CH_2)_2{\cdot}NH\ Et$ (XIX)

ethanol with thionyl bromide[29] under much milder conditions than with hydrobromic acid failed to improve the yield of the base (XIX), the low yield probably being due to the preferential cyclisation of 2-diethylaminoethoxyethyl bromide in the presence of ethylamine.

NN-Diethyldiglycollamic acid (XXIII) required for the condensation with *N*(2-diethylaminoethoxyethyl) ethylamine, was prepared by hydrolysis of methyl *NN*-diethyldiglycollamate with ethanolic potassium hydroxide. The product, obtained after evaporation of the ethanol, acidification extraction and distillation, had a high equivalent weight, and could not be separated into its components by fractionation or by chromatography on a charcoal-cellulose column. Normal two-phase extraction procedures were also incapable of effecting a separation of acidic and neutral fractions of the product, due to their high water-solubility. A satisfactory separation was, however, effected by dissolving in dry ether, and precipitating acid material with dry ammonia gas. Evaporation of the ether gave a neutral oil which was identified as *NNN′N′*-tetra-ethyldiglycolldiamide (XX). The precipitated ammonium salts were only partially soluble in ethanol, the insoluble material being identified as ammonium diglycollate (XXI). Precipitation of the ethanol-soluble salt with ether gave ammonium *NN*-diethyldiglycollamate (XXII). Purification of the latter, however, proved difficult, owing to its partial

dissociation in the hot ethanol used for recrystallisation, which resulted in loss of ammonia and consequent contamination of the salt by traces of

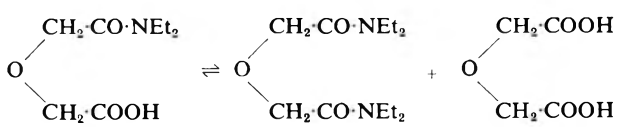
$Et_2N{\cdot}CO{\cdot}CH_2O{\cdot}CH_2{\cdot}CO{\cdot}NEt_2$ (XX)

$NH_4{\cdot}O{\cdot}CO{\cdot}CH_2{\cdot}O{\cdot}CH_2{\cdot}CO{\cdot}O{\cdot}NH_4$ (XXI)

$Et_2N{\cdot}CO{\cdot}CH_2{\cdot}O{\cdot}CH_2{\cdot}COO{\cdot}NH_4$ (XXII)

the more-soluble parent acid. Decomposition of the almost pure ammonium *NN*-diethyldiglycollamate with dilute hydrochloric acid, yielded the required *NN*-diethyldiglycollamic acid (XXIII).

At this stage no satisfactory explanation could be advanced for the formation of *NNN'N'*-tetraethyldiglycolldiamide (XX) and diglycollic acid as by-products in the preparation of *NN*-diethyldiglycollamic acid by hydrolysis of pure methyl *NN*-diethyldiglycollamate. Attention was therefore turned to the preparation of *NN*-diethyldiglycollamic acid by an alternative route, the reaction of diglycollic anhydride with diethylamine. Direct condensation under reflux in the absence of solvent followed by distillation gave an impure product, which, when dissolved in ether and treated with ammonia as described above, gave the same three products obtained in the first method. Similar results were also obtained when the distillation step was omitted, suggesting that *NNN'N'*-tetraethyldiglycolldiamide and diglycollic acid are formed as by-products of the condensation reaction, though this was shown subsequently not to be the case. Thus condensation of diglycollic anhydride with diethylamine under much milder conditions, using benzene as solvent and isolation of the product, without prior distillation, by precipitation of the ammonium salt, gave the required *NN*-diethyldiglycollamic acid, and only trace amounts of diglycollic acid and its bis-diethylamide. That these by-products are formed in all the above reactions by the following disproportionation at elevated temperatures was demonstrated by distilling *NN*-diethyldiglycollamic acid under vacuum, and examining the distillate by the ammonium salt separation technique. All three products were found to be present, *NNN'N'*-tetraethyldiglycolldiamide being formed to the extent of approximately 20 per cent. The separation method is not suitable for a more

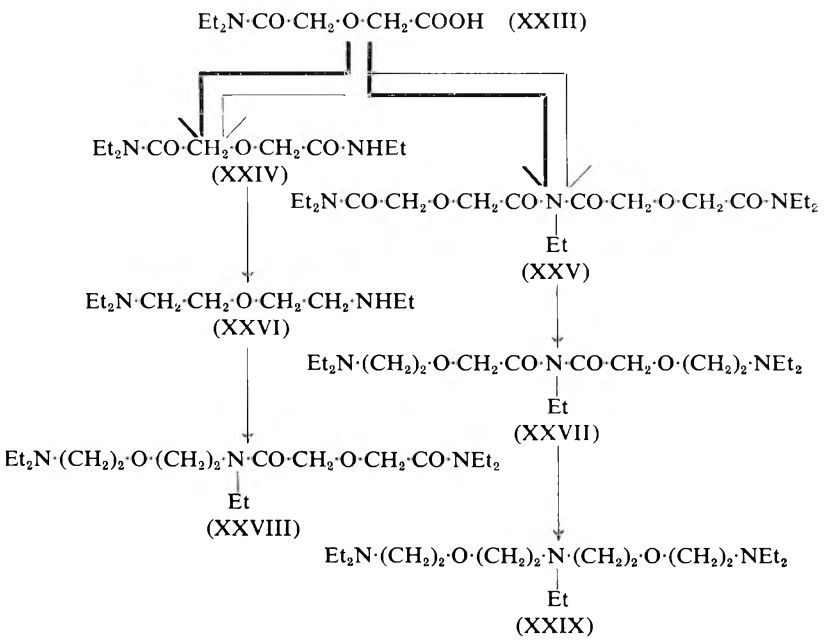


detailed study of the disproportionation owing to the partial dissociation of both ammonium diglycollate and ammonium *NN*-diethyldiglycollamate.

Analogous disproportionations, described by Fourneaux and Sabetay[30,31], have been used for the preparation of the monoesters of succinic, glutaric, adipic, azelaic, sebacic and similar dibasic acids. The acids when heated for several hours with the corresponding di-ester reach an equilibrium with the monoester, which is present to the extent of 20 to 30 per cent. The position of the equilibrium, which can also be reached from the monoester, appears to vary with the nature of the acid,

the time of heating and the temperature. So far as we are aware such acidolysis type reactions have not been reported previously in the preparation of the half amides of dibasic acids, and were not observed in the preparation either of *NN*-diethylglutaramic acid (present work) or of *NN*-diethyladipamic and *NN*-di-n-propyladipamic acids[25]. Anschütz and Jaeger[28], however, have reported the formation of dimethyl diglycollate as a by-product in the preparation of methyl hydrogen diglycollate from diglycollic anhydride and methanol, which suggests that such disproportionations are facilitated by the ether link in the diglycollic acid series. The significantly lower dissociation constants of diglycollic acid (pK_1 2·96, pK_2 4·43) compared with glutaric acid (pK_1 4·32, pK_2 5·54) suggest that the mechanism may perhaps be ionic as in the Cannizzaro and related reactions.

NN-Diethyldiglycollamic acid (XXIII) was used to prepare *N*-(2-diethylaminoethoxyethyl) ethylamine in improved yield as outlined below. Conversion to the acid chloride and reaction with excess ethylamine gave *NNN'*-triethyldiglycolldiamide (XXIV) together with some bis-(*NN*-diethyldiglycollamoyl) ethylamine (XXV). Reaction of the acid



chloride with ethylamine in the presence of triethylamine, on the other hand, gave mainly the base (XXV). The two amides were not readily separated by fractional distillation, but reduction of the mixture with lithium aluminium hydride yielded the required *N*-(2-diethylaminoethoxyethyl) ethylamine (XXVI), which was readily separated by fractionation from the accompanying *NN*-bis(2-diethylaminoethoxyacetyl) ethylamine (XXVII). Condensation of the base (XXVI) with *NN*-diethyldiglycollamoyl chloride, gave *NNN'*-triethyl-*N'*-(2-diethylaminoethoxyethyl)

diglycolldiamide (XXVIII), which on reduction with lithium aluminium hydride yielded *NN*-bis-(2-diethylaminoethoxyethyl) ethylamine (XXIX). Quaternisation of the latter with methyl and ethyl iodides gave the required tris-onium compounds (XIII, R′ = Et, R = Me and Et).

The resistance of the imide link in *NN*-bis(2-diethylaminoethoxyacetyl)-ethylamine (XXVII) to reduction by lithium aluminium hydride is typical of such compounds. Phthalimide is reduced, in unstated yield, only after 28 hours refluxing with the reagent in ether[32]. Prolonged reduction of the imide (XXVII) with lithium aluminium hydride, similarly gave *NN*-bis-(2-diethylaminoethoxyethyl) ethylamine (XXIX) in 35 per cent yield.

The polymethylene tris-onium compounds (XIV, R = Me and Et) were obtained by quaternisation of *NN*-bis-(5-diethylaminopentyl)ethylamine, which was prepared from ethyl hydrogen glutarate by methods already described for the preparation of *NN*-bis-(6-diethylaminohexyl)ethyl-amine[25].

1,19-Bis-diethylamino-7,13-diethyl-7,13-diazanonadecane and 1,19-bis-diethylamino-7,13-diethyl-7,13-diaza-10-oxanonadecane, intermediates in the preparation of the tetra-onium compounds (XVI, R = Me and Et) and (XV, R = Me and Et), were obtained from glutaric and diglycollic acids respectively, and 6-diethylaminohexyl-ethylamine by methods already described[26].

Experimental

Ethyl NN-*diethylglutaramate* was prepared from ethyl hydrogen glutarate (93·5 g.) by the method described for the preparation of ethyl *NN*-diethyladipamate.[25] *Ethyl* NN-*diethylglutaramate* was obtained as a yellow oil (114·2 g., 91 per cent), b.p. 120–124°/0·3 mm., n_D^{25} 1·4520. Found: N, 6·2 per cent. $C_{11}H_{21}NO_3$ requires N, 6·5 per cent.

5-*Hydroxpentyldiethylamine* was prepared by lithium aluminium hydride reduction of ethyl *NN*-diethylglutaramate (89 g.) and was obtained as a colourless oil (60·1 g., 91·3 per cent)[24], b.p. 90°/0·55 mm. n_D^{21} 1·4528.

5-*Diethylaminopentylethylamine* was prepared from 5-hydroxypentyl-diethylamine by the method described for the preparation of 6-diethyl-aminohexylethylamine[25]. The *product* was obtained as a colourless oil (6·8 g., 12 per cent), b.p. 68–70°/0·25 mm., $n_D^{24·5}$ 1·4428.

NN-*Diethylglutaramic acid* was prepared from ethyl *NN*-diethyl-glutaramate (24·5 g.) by the method described for the preparation of *NN*-diethyladipamic acid[25]. NN-*Diethylglutaramic acid* was obtained as a yellow viscous oil (11·3 g., 54 per cent), b.p. 173–175°/0·45 mm., n_D^{24} 1·4747. Found: N, 7·5 per cent. $C_9H_7NO_3$ requires N, 7·5 per cent.

Bis-5-*diethylaminopentylethylamine* was prepared from *NN*-diethyl-glutaramic acid (4·8 g.) and excess 5-diethylpentylethylamine (6·7 g.) by the method described for the preparation of bis-6-diethylaminohexyl-ethylamine[25].

Bis-5-*diethylaminopentylethylamine* was obtained as a yellow oil (2·4 g., 33 per cent), b.p. 147–150°/0·25 mm., n_D^{23} 1.4585. Found: N, 12·8 per cent. $C_{20}H_{45}N_3$ requires N, 12·8 per cent.

Diglycollic anhydride was prepared from diglycollic acid (269 g.) by refluxing with acetic anhydride (525 g.) for 4 hours. Acetic acid was removed by distillation, and the product refluxed with acetic anhydride (242 g.) for a further 2 hours. Removal of excess acetic anhydride and distillation gave the product as a colourless crystalline solid (220 g., 94·4 per cent), b.p. 130–131°/20 mm., m.p. 97°. Hurd and Glass[27] gave b.p. 130°/20 mm.

Methyl hydrogen diglycollate. Diglycollic anhydride (186 g.) was refluxed with methanol (65 ml.) for 2 hours, and the product fractionated to yield methyl hydrogen diglycollate (175·6 g., 74 per cent), b.p. 176°/14 mm., n_D^{23} 1.4450. Anschutz and Jaeger[28] gave b.p. 173–174°/12 mm.

Methyl NN-*diethyldiglycollamate.* Methyl hydrogen diglycollate (175·6 g.) in thionyl chloride (150 ml.) was heated to reflux for 1½ hours, and the excess reagent removed under reduced pressure. The acid chloride without further purification, in ether (300 ml.) was added (45 minutes) to a stirred solution of diethylamine (280 ml.) in ether (1 l.). The diethylamine hydrochloride was removed by filtration, the filtrate dried (Na_2SO_4), the solvent distilled, and the product fractionated to yield *methyl* NN-*diethyldiglycollamate* as a pale yellow oil (200 g., 83 per cent), b.p. 128–130°/0·55 mm., $n_D^{23\cdot5}$ 1·4583. Found: N, 6·8. $C_9H_{17}NO_4$ requires N, 6·9 per cent.

NN-*Diethyldiglycollamic acid.* (*a*) Methyl *NN*-diethyldiglycollamate (198·2 g.) was refluxed for 30 minutes with ethanolic potassium hydroxide (1,889 ml.; 1·033 0·5N). After neutralisation of the excess alkali with dilute hydrochloric acid (100 ml.; 1·084 0·5N), the bulk of the ethanol was removed by distillation, the solution acidified with dilute hydrochloric acid (172 ml.; 5N) and concentrated under reduced pressure. The solution was extracted with benzene, the solution dried (Na_2SO_4), the solvent evaporated and the product distilled to yield a yellow oil (156 g.), b.p. 180–187°/0·3 mm., n_D^{18} 1·4759, containing some solid material. Found: equiv. 237·8 ($C_8H_{15}NO_4$ requires, equiv. 189·2). The crude product (54 g.) was dissolved in ether (1 l.) and dry ammonia gas bubbled through the solution for 20 minutes, to yield a precipitate of mixed ammonium salts (37·2 g.). Evaporation of the filtrate and distillation of the product yielded NNN′N′-*tetraethyldiglycolldiamide* as a pale yellow oil (21·5 g.) b.p. 159–160°/0·08 mm., n_D^{20} 1·4777. Found: N, 11·6 per cent. $C_{12}H_{24}N_2O_3$ requires N, 11·5 per cent.

The mixed ammonium salts were refluxed gently with ethanol (300 ml.), and the solution cooled, and filtered to yield the insoluble crude *ammonium diglycollate*, m.p. 225–226° (decomp.). Concentration of the ethanolic solution and addition of ether gave a colourless crystalline precipitate of *ammonium* NN-*diethyldiglycollamate* (29·5 g.), m.p. 125°–126°. Found: N, 13·8. $C_8H_{18}NO_4$ requires N, 13·6 per cent.

Ammonium *NN*-diethyldiglycollamate (29 g.) was acidified with dilute hydrochloric acid (142 ml.; 0·9945N), and the solution extracted with benzene to yield NN-*diethyldiglycollamic acid* as a yellow viscous oil (20·5 g.), n_D^{24} 1·4743, which slowly crystallised on standing to yield an almost colourless solid m.p. 68°. Found: N, 7·3; equiv. 188·9. $C_8H_{15}NO_4$

requires N, 7·4 per cent; equiv. 189·2. S-*Benzylthiuronium salt*, m.p. 150–151°. Found: N, 11·65. $C_{16}H_{25}N_4O_2S$ requires N, 11·8 per cent.

(*b*) Diethylamine (25 ml.) was slowly added to diglycollic anhydride (25 g.) and the mixture permitted to reflux, without the external application of heat, until the anhydride had completely dissolved (45 minutes). The mixture was refluxed for a further 30 minutes, and excess diethylamine then removed by evaporation under reduced pressure. The crude product was dissolved in ether (250 ml.) and dry ammonia gas bubbled through the solution for 60 minutes, to yield a precipitate of mixed ammonium salts. Evaporation of the filtrate yielded a neutral oil (11·7 g.), which on distillation gave *NNN'N'*-tetraethyldiglycolldiamide b.p. 160°/0·08 mm. $n_D^{17.5}$ 1·4792. Found: N, 11·4. Calc. for $C_{12}H_{24}N_2O_3$, N, 11·5 per cent.

The mixed ammonium salts were gently heated with dry ethanol (50 ml.), and the solution cooled and filtered to yield ammonium diglycollate (6·9 g.) m.p. 225–226° (decomp.). Concentration of the ethanolic solution and addition of ether gave a precipitate of ammonium *NN*-diethyldiglycollamate (15·1 g.), m.p. 125°. Evaporation of the mother liquors yielded an acid oil (12·1 g.), being a mixture of ammonium *NN*-diethyldiglycollamate and *NN*-diethyldiglycollamic acid. *NN*-Diethyldiglycollamic acid (9·3 g.) was obtained by decomposition of the ammonium *NN*-diethyldiglycollamate by the method described above.

(*c*) Diethylamine (150 ml.) in dry benzene (300 ml.) was run into an ice-cold solution of diglycollic anhydride (70 g.) in dry benzene (250 ml.), the mixture slowly heated and refluxed for 3 hours. Evaporation of the solvent and excess diethylamine under reduced pressure gave an oil, which was dissolved in dry benzene (400 ml.). Dry hydrogen chloride was passed into the solution until a precipitate of diethylamine hydrochloride was obtained (ca. 20 min.). The precipitate was removed by filtration, and the filtrate treated for a further 5 minutes with dry hydrogen chloride. Excess HCl was removed by passing a stream of air through the solution heated to about 30°, solvent lost by evaporation being made up by further additions (3 × 150 ml.) from time to time. The solution was cooled in ice and salt, filtered from diethylamine hydrochloride, dried (Na_2SO_4), and evaporated to yield a golden brown viscous oil, which formed a brownish-white solid (93·5 g., 82 per cent) on standing. The solid was gently melted and triturated with dry ether (100 ml.) to yield *NN*-diethyldiglycollamic acid as a fine white solid, which was filtered and dried over P_2O_5 *in vacuo*. The product which was not completely pure, had equiv. 204·6, and was used in the next stage. An analytical sample was obtained by dissolving the acid (1 g.) in distilled water (20 ml.) and passed through a column of Zeocarb 225 (column dimensions 12 in. × 1¼ in., 120 g.) in its H^+ form. Evaporation of the eluate (800 ml.), yielded a clear oil which readily crystallised on cooling to yield *NN*-diethyldiglycollamic acid monohydrate, m.p. 68–69°. Found: N, 6·5; equiv. 206·1. $C_8H_{15}NO_4.H_2O$ requires N, 6·76; equiv. 207·2. Over P_2O_5 the monohydrate becomes oily, but recrystallises on exposure to air.

Disproportionation of NN-*diethyldiglycollamic acid.* *NN*-Diethyldiglycollamic acid (11 g.), 95–98 per cent pure, obtained from previous

experiments by the ammonium salt precipitation was distilled, to yield a homogeneous distillate (9·65 g.) b.p. 160–162°/0·06 mm, n_D^{22} 1·4757, equiv. 221·6. The products separated by precipitation from ether with ammonium as described above yielded *NNN'N'*-tetraethyldiglycolldiamide (1·90 g.), ammonium diglycollate (0·940 g.) and *NN*-diethyldiglycollamic acid (3·8 g.) equiv. 194 (required 189·2). A further yield of crude *NN*-diethyldiglycollamic acid (1·075 g.) was also obtained.

2-*Diethylaminoethoxyethanol.* Methyl *NN*-diethylglycollamate (76·6 g.) was reduced with lithium aluminium hydride as described for the preparation of 6-hydroxydiethylamine[33] to yield 2-diethylaminoethoxyethanol (53 g., 88 per cent) as a colourless oil, b.p. 79°/0·5 mm., n_D^{22} 1·4475. Found: N, 8·9; equiv. 160·1. Calc. for $C_8H_{19}NO_2$, N, 8·7 per cent; equiv. 161·2. Horne and Schriner[34] gave b.p. 92–95°/7 mm.

N-(2-*Diethylaminoethoxyethyl*)*ethylamine.* (*a*) *N*-(2-Diethylaminoethoxyethyl)ethylamine was prepared from 2-diethylaminoethoxyethanol (12·7 g.) by the method described for the preparation of 6-*n*-propylhexyldi-*n*-propylamine[25]. The *product* was obtained as a colourless oil (3·2 g., 21·6 per cent), b.p. 74°/0·45 mm., $n_D^{19·5}$ 1·4455.

(*b*) 2-Diethylaminoethoxyethanol (39·1 g.) in benzene (200 ml.) was stirred while a solution of thionyl bromide[29] (50·6 g.) in benzene (50 ml.) was added (40 min.). Evaporation of the reaction mixture under reduced pressure yielded a dark brown viscous liquid, which failed to crystallise. The liquid was dissolved in ethanol (100 ml.), and added (10 min.) to a refluxing solution of ethylamine (100 ml.) in ethanol (100 ml.), and the mixture refluxed for 1 hour. Evaporation of the liquid, basifying and extraction with ether yielded N-(2-*diethylaminoethoxyethyl*)*ethylamine* as a colourless oil (2·6 g., 5·8 per cent), b.p. 76°/0·5 mm., n_D^{21} 1·4470.

(*c*) *NN*-diethyldiglycollamic acid (38·3 g.) in benzene (20 ml.) was refluxed with thionyl chloride (20 ml.) for 15 minutes, and evaporated under reduced pressure. The acid chloride in benzene (120 ml.) was added slowly (25 min.) to a stirred solution of ethylamine (120 ml.) in benzene (500 m.). After evaporation to dryness under reduced pressure it was extracted with ether, filtered (to remove ethylamine hydrochloride) and dried (Na_2SO_4). On removal of solvent the product was distilled at 100–166°/0·45 mm. (35 g.). Lithium aluminium hydride reduction of the mixed amides and fractionation of the product yielded crude N-(2-*diethylaminoethoxyethyl*)-*ethylamine* b.p 78–82°/0·4 mm. (9·59 g., 25·2 per cent) n_D^{21} 1·4470. Continued fractionation yielded NN-*bis*-(2-*diethylaminoethoxyacetyl*)-*ethylamine*, b.p. 105/0·35 mm. (9·4 g.), $n_D^{25·5}$ 1·4713. Found equiv. (titration) 180·2. $C_{18}H_{37}N_3O_4$ requires equiv. 179·8.

NN-*Bis*-(2-*diethylaminoethoxyethyl*)*ethylamine.* (*a*) *NN*-Bis-(2-diethylaminoethoxyethyl)ethylamine was prepared from *NN*-diethyldiglycollamic acid (4·6 g.) and *N*-(2-diethylaminoethoxyethyl)ethylamine (4·6 g.) by the method described for the preparation of bis-(6-di-*n*-propylaminohexyl)-n-propylamine[25], and was obtained as a pale yellow *oil* (1·5 g., 19 per cent), b.p. 140–142°/0·3 mm., $n_D^{22·5}$ 1·4567. Found: N, 12·5. $C_{18}H_{41}N_3O_2$ requires N, 12·7 per cent.

(*b*) *NN*-Bis-(2-diethylaminoethoxyacetyl)ethylamine (14·3 g.) in ether (20 ml) was added (10 min.) to a stirred refluxing solution of lithium aluminium hydride (3 g.) in ether (200 ml). After refluxing for 14 hours and standing for 32 hours at room temperature the mixture was worked up and extracted in the usual manner to yield an oil, b.p. 80–130° (bath)/0·75 mm. (9·5 g.). Further extractions with benzene and ether yielded only a few more drops of oil. Fractionation of the product yielded (1) NN-*Bis-(2-diethylaminoethoxyethyl)ethylamine* as a pale yellow oil, b.p. 84–90°/0·75 mm. (5 g.), n_D^{21} 1·4645. Found equiv. (titration) 114·0. $C_{18}H_{41}N_3O_2$ requires equiv. 110·5. (2) Unchanged *NN*-bis-(2-diethylaminoethoxyacetyl)ethylamine b.p. 101–105°/0·7 mm. (3·2 g.), n_D^{21} 1·4660. Found equiv. (titration) 205·6. $C_{18}H_{37}N_3O_4$ requires equiv. 179·8.

NNN-*Tris-onium Compounds* were prepared from either *bis*-(5-diethylaminopentyl)ethylamine or *NN*-bis-(2-diethylaminoethoxyethyl)ethylamine by refluxing with the appropriate alkyl halide in ethanol, evaporation of the solvent and crystallisattion. Reflux time, crystallisation solvent and yields are indicated for each compound in that order, in parenthesis.

6,6-*Diethyl*-6-*azoniaundecylenebis*-(*triethylammonium*)*tri-iodide* (45 min., ethanol-ether; 36 per cent), m.p. 273–274°. Found: N, 5·1; I, 47·9. $C_{26}H_{60}I_3N_3$ requires N, 5·3; I, 47·9 per cent.

6-*Ethyl*-6-*methyl*-6-*azoniaundecylenebis*-(*diethylmethylammonium*) *tri-iodide* (10 min.; ethanol-methanol; 64 per cent), m.p. 266°. Found: N, 5·4; I, 50·6. $C_{23}H_{54}I_3N_3$ requires N, 5·6; I, 50·5 per cent.

6-*Ethyl*-6-n-*propyl*-6-*azoniaundecylenebis*-(*diethyl*-n-*propylammonium*) *tri-iodide* (60 min.; acetone-ethanol-ether; 17 per cent), m.p. 221–222°. Found: N, 5·0; I, 45·1. $C_{29}H_{66}I_4N_4$ requires N, 5·0; I, 45·45 per cent.

6,6-*Diethyl*-3,9-*dioxa*-6-*azoniaundecylenebis*(*triethylammonium*) *tri-iodide* (30 min.; ethanol-methanol; 32 per cent), m.p. 255°. Found: N, 5·3; I, 47·2. $C_{24}H_{56}I_3N_3O_2$ requires N, 5·3; I, 47·6 per cent.

6-*Ethyl*-6-*methyl*-3,9-*dioxa*-6-*diazoniaundecylenebis* (*diethylmethylammonium*) *tri-iodide* (15 min.; methanol; 71 per cent), m.p. 262–263°. Found: N, 5·6; I, 50·1. $C_{21}H_{50}I_3N_3O_2$ requires N, 5·5; I, 50·3 per cent.

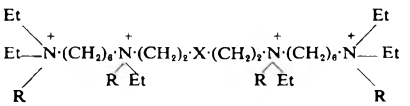
1,19-*Bis*-(*diethylamino*)-7,13-*diethyl*-7,13-*diazanonadecane* was prepared from glutaric acid (2·7 g.) and excess 6-diethylaminohexylethylamine (16·7 g.) by the method described for the preparation of 1,20-bisdiethylamino-7,14-diethyl-7,14-diazaeicosane[26]. 1,19-*Bis*-(*diethylamino*)-7,13-*diazanonadecane* was obtained as a pale yellow oil (4·5 g., 43·4 per cent), b.p. 250–255° (bath 0·55 mm.). Found: N, 11·8; equiv. 116·4. $C_{29}H_{64}N_4$ requires N, 11·9 per cent; equiv. 117·2.

1,19-*Bis*-(*diethylamino*)-7,13-*diethyl*-7,13-*diaza*-10-*oxanonadecane* was prepared from diglycollic acid (3·3 g.) and excess 6-diethylaminohexylethylamine (19·9 g.) by the method described for the preparation of 1,20-bis-(diethylamino)-7,14-diethyl-7,14-diazaeicosane[26]. 1,19-*Bis*-(*diethylamino*)-7,13-*diethyl*-7,13-*diaza*-10-*oxanonadecane* was obtained as a pale yellow oil (4·7 g., 40·6 per cent), b.p. 240–250° (bath)/0·3 mm., n_D^{20} 1·4639. Found: N, 11·9; equiv. 119·4. $C_{28}H_{62}N_4O$ requires N, 11·9 per cent; equiv. 117·7.

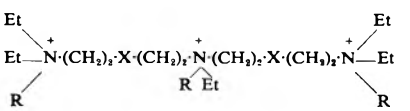
NNNN-*Tetra-onium compounds* were prepared from either 1,19-bis-(diethylamino)-7,13-diethyl-7,13-diazanonadecane or 1,19-bis-(diethylamino)-7,13-diethyl-7,13-diaza-10-oxanonadecane by refluxing with the appropriate alkyl halide in ethanol, evaporation of the solvent and crystallisation. Reflux time, crystallisation solvent and yields are indicated for each compound in that order, in parenthesis.

TABLE I

RELATIVE MOLAR POTENCIES OF XIII A AND B; XIV A, B AND C; XV A, B AND C AND XVI A, B AND C



Compound	R	X	Relative molar potencies				
			Cat	Rabbit	Chick	Mouse	Frog
XV A	Et	O	278	108	139	165	375
XVI A	Et	CH_2	417	125	230	174	208
XV B	Me	O	87	66	110	132	77
XVI B	Me	CH_2	110	82	158	165	26
XV C	Pr	O	234	96	242	146	63
XVI C	Pr	CH_2	263	110	242	151	72



XIII A	Et	O	5	5	No paralysis with 5 mg./kg.	*	*
XIV A	Et	CH_2	50	*	101	22	*
XIII B	Me	O	No paralysis with 2 mg./kg.	*	16	No paralysis with 20 mg./kg.	*
XIV B	Me	CH_2	6	*	52	11	*
XIV C	Pr	CH_2	2	*	88	13	*

* Insufficient material.

7,7,13,13-*Tetra-ethyl*-7,13-*diazonianonadecylenebis-(triethylammonium) tetra-iodide* (25 min.; ethanol; 98 per cent), m.p. 264°. Found: N, 5·0; I, 46·2. $C_{37}H_{84}I_4N_4$ requires N, 5·1; I, 46·4 per cent.

7,13-*Diethyl*-7,13-*dimethyl*-7,13-*diazonianonadecylenebis-(diethylmethyl ammonium) tetra-iodide* (10 min.; ethanol-methanol; 98 per cent), m.p. 236–237°. Found: N, 5·3; I, 48·8. $C_{33}H_{76}I_4N_4$ requires N, 5·4; I 48·95 per cent.

7,13-*Diethyl*-7,13-*di-n-propyl*-7,13-*diazonianonadecylenebis-(diethyl-n-propylammonium) tetra-iodide* (50 min.; acetone-ethanol-ether; 24 per cent), m.p. 175–176°. Found: N, 4·9; I, 43·6. $C_{41}H_{92}I_4N_4$ requires N, 4·9; I, 44·2 per cent.

7,7,13,13-*Tetraethyl*-7,13-*diazonia*-10-*oxanonadecylenebis*-(*triethylammonium*) *tetra-iodide* (25 min.; ethanol; 54 per cent), m.p. 236°. Found: N, 5·2; I, 46·4. $C_{36}H_{82}I_4N_4O$ requires N, 5·1; I, 46·3 per cent.

7,13-*Diethyl*-7,13-*dimethyl*-7,13-*diazonia*-10-*oxanonadecylenebis*-(*diethyl methylammonium*) *tetra-iodide* (10 min.; ethanol-ether; 72·5 per cent), m.p. 210–211°. Found: N, 5·4; I, 48·5. $C_{32}H_{74}I_4N_4O$ requires N, 5·4; I, 49·0 per cent.

Pharmacological Methods and Results

The experimental methods and the materials have been described elsewhere[26,43,44]. The results are shown in Table I, which sets out the comparative molar potencies. All the compounds showed muscle-relaxant properties of the tubocurarine type without depolarising activity. The tetra-onium compounds (XV A, B and C) which contained only one ether link were much more potent than the tris-onium derivatives (XIII A and B) which contained two such linkages. The former were usually more potent than tubocurarine itself.

The results show therefore that the non-ether compounds were more potent than the analogous ethers and that introduction of a second ether link further lowered potency. The tetra-onium compounds showed no ganglion-blocking activity when tested on the nictitating membrane of the cat or the guinea pig ileum; the tris-onium derivatives when tested on these preparations showed some ganglion-blocking activity but less than that of tubocurarine. Thus there was no significant fall in blood pressure when muscle-relaxant doses were given by intravenous injection into pento-barbitone-anaesthetised cats.

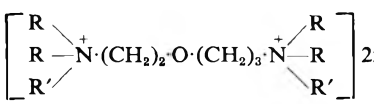
Discussion

The results show that replacement of a methylene group ($-CH_2-$) of the interonium-polymethylene chain by an ether link lowers neuromuscular-blocking potency in almost every compound. If it can be assumed that in our compounds repulsion of like charges on the onium nitrogen results in the chains being maximally extended then replacement of a $-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-$ link by a $-\overset{|}{\underset{|}{C}}-\overset{|}{O}-\overset{|}{\underset{|}{C}}-$ link reduces chain length by 0·12Å. This change seems inadequate to explain the fall in potency observed.

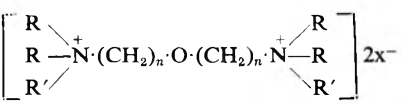
Other factors to be considered include solubility changes, which may modify tissue distribution, and the possibility of increased bonding at "sites of loss". Actual solubilities have not been recorded, but our experience of diglycollic acid derivatives and related substances, leads us to believe that the water-solubility of poly-onium compounds will be enhanced by the presence of the ether link. In view of the already high water-solubility of the corresponding polymethylene compounds it seems unlikely that this factor is significant in itself, though it could alter the balance of tissue distribution. The possibility that ether-linked compounds suffer increased binding at sites of loss may be determined by the study of absorption, distribution and excretion in the intact animal,

though this seems unlikely in view of the extreme range of ganglion-blocking potency shown by the closely related onium-ethers (XXX) and (XXXI) discussed below[35–38]. The study of such physical properties as solubility, distribution and adsorption characteristics in corresponding ether and polymethylene compounds should, however, throw light on this point.

If it is assumed, in the absence of direct evidence to the contrary, that structural differences between ether-linked and polymethylene-linked polyonium compounds are unimportant for concentration at the site of action, then questions of drug-receptor binding and intrinsic activity[39,40] are relevant. In this connection the ideas of Fakstorp and Pedersen[37] seem worthy of further consideration. Thus it was suggested that in a series of bis-choline ethers, which they examined for ganglion-blocking activity, proximity of the ether oxygen to the quaternary nitrogen might cause an electron drift from the oxygen which would then assume a partial positive charge. This concept was used to explain the more stable attachment to the negatively charged "berth" of the esteratic site in the conventional receptor model[41] in the compounds (XXX, when R = R′ = Me; R = Me, R′ = Et; and R = Et, R′ = Me) which are more active than hexamethonium.



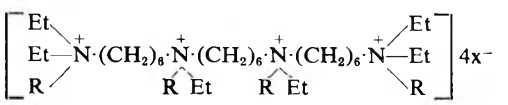
(XXX)



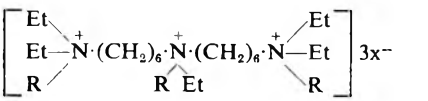
(XXXI)

This electron drift, however, is only likely to be significant where at the most one or two carbon atoms separate the –O– and –$\overset{+}{N}$– links, and will tend to be nullified when the ether link is situated symmetrically with respect to the two onium groups. It might be argued, therefore, that the enhanced availability of electrons contributes to the fall in potency seen in the analogous compounds of structure (XXXI, where n = 2 and 3), but it must be emphasised that unequivocal conclusions are not possible because changes of inter-onium distance are also involved. This criticism, however, does not apply to the comparison of our own ether-linked compounds with the corresponding polymethylene derivatives, and the observed reduction in potency could be due to an anti-bonding effect arising from the availability of electrons on the symmetrically linked ether oxygen.

The tetra-onium compounds (XVI A, B and C) are more potent than the analogous trihexatetrazonium compounds (XXXII, R = Et, Me and n-Pr)[26,42], but the tris-onium compounds (XIV, R = Me and Et) are less potent than the analogous compounds (XXXIII, R = Me and Et)[25].



(XXXII)



(XXXIII)

In all four series of compounds [(XIII), (XIV), (XV) and (XVI)] examined, subsitution of any one *N*-ethyl by an *N*-methyl or *N*-propyl substituent reduces activity. This strengthens the view that optimum receptor fit at the anionic site is to be obtained with polyonium compounds when all groups on the onium atom are ethyl[25,26].

Acknowledgement. We thank Mr. Peter Leitch for technical assistance and the Department of Scientific and Industrial Research for a maintenance allowance to one of us (D. E. M.).

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THE EFFECT OF THALIDOMIDE IN EXPERIMENTAL GASTRIC ULCERS

By K. Martindale, G. F. Somers and C. W. M. Wilson

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Received May 23, 1960

A study has been made of the effects of thalidomide, a new sedative hypnotic drug, in stress and phenylbutazone-induced ulcers in the rat. The results have shown that thalidomide inhibits stress induced ulcers but does not affect the local mechanisms involved in the pathogenesis of phenylbutazone-induced ulcers. It does eliminate stress-induced components in phenylbutazone ulcers, which indicates that the mode of action is a central one.

Neurological stimuli play an important part in the aetiology of peptic ulcers, and sedative drugs like phenobarbitone are routinely used in their treatment. It was of interest therefore to examine the value of thalidomide (Distaval)[1], a new sedative hypnotic drug, in experimental gastric ulcers before clinical trial.

Experimental

Ulcers were induced in rats by stress and by injection of phenylbutazone.

Stress Ulcers

These were produced by a modification of the method of Rossi, Bonfils, Lieffoogh and Lambling[2]. Male albino rats (Agricultural Research Council, Compton) weighing approximately 150–200 g. were housed in cages with wire floors to prevent access to sawdust and faeces. They were deprived of food for 24 hours but water was always supplied. Their thoraces and abdomens were then enclosed in Plaster of Paris bandage B.P.C. for 24 hours, food still being withheld, after which the rats were killed for pathological examination. At post-mortem the stomachs were removed between ligatures, washed and filled with formal saline through the oesophageal junction and examined by transmitted light. The stomachs were then opened for closer examination and specimens taken for histological examination. The degree of ulceration was scored according to the following arbitrary scale.

Score 0 .. Normal stomach
Score 0·5 .. Grey discolouration and thinning of the mucosa
Score 1·0 .. Petechial haemorrhages or minute pin point ulcers
Score 2·0 .. One or two small ulcers
Score 3·0 .. Many ulcers
Score 4·0 .. Perforated ulcers

For *prophylaxis*, thalidomide was administered orally as a suspension in water and gum tragacanth before the application of the plaster and again after 12 hours. For *curative effects*, it was first administered 24 hours after the application of the plaster when ulcers had developed in the

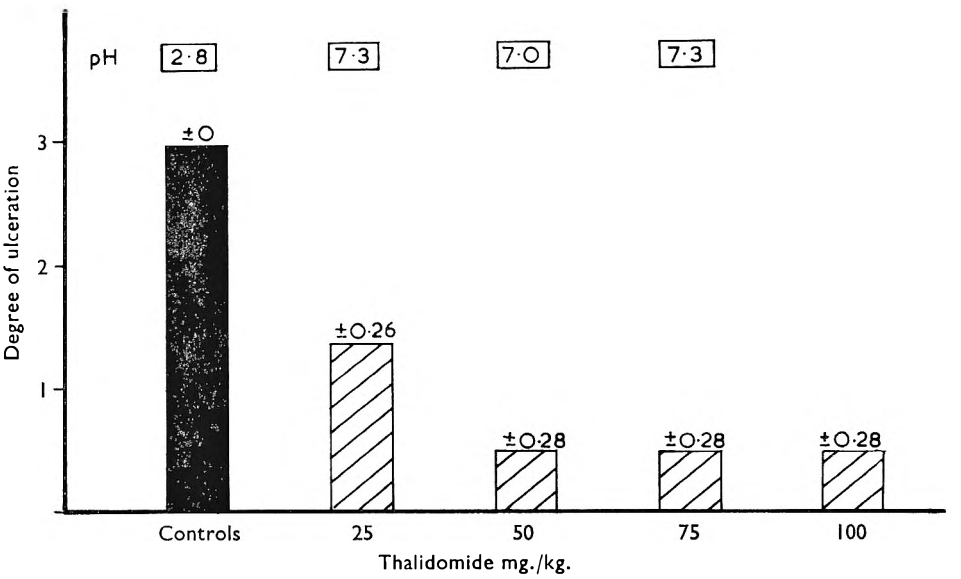

FIG. 1. Anti-ulcerative activity and effect on gastric acidity of thalidomide in partially restrained rats. Each column is the mean with standard error from 4 rats.

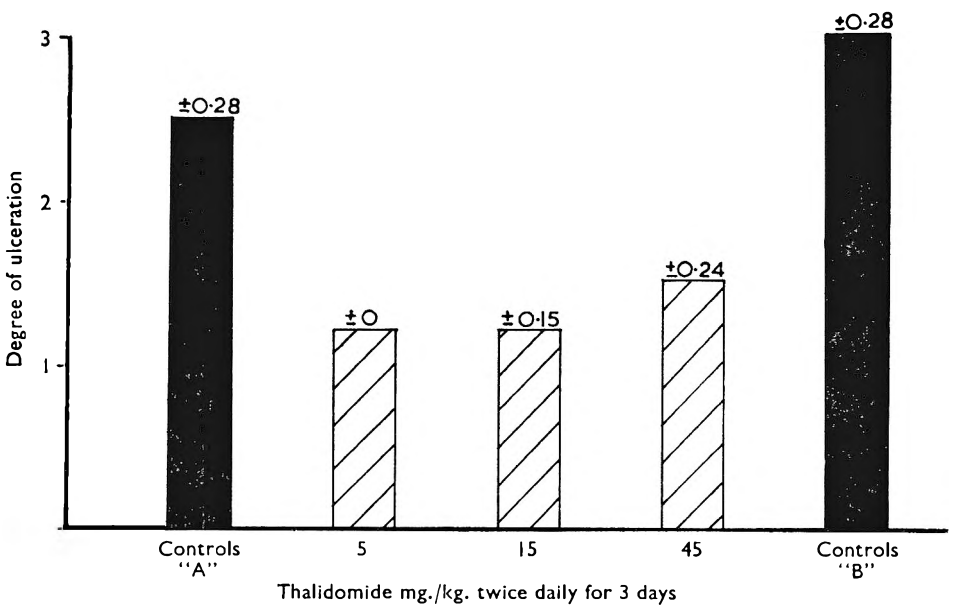

FIG. 2. Curative effect of thalidomide in rats ulcerated by restraint. Each column is the mean with the standard error from 4 rats.

control groups. It was repeated twelve hourly over three consecutive days after which the rats were killed for examination as before. These rats were maintained on sucrose.

Phenylbutazone Ulcers

These were produced by the intramuscular injection of a range of doses of phenylbutazone. Control groups were used in all experiments in a 1 : 1 ratio as recommended by Bonfils and others[3], and the experimental conditions were carefully standardised to avoid external stress. Different sized groups of male rats weighing between 150 and 200 g. were placed in uniform cages for 24 hours before the experiment commenced. They were fed on a pellet diet 41B (J. Rank & Co. Ltd.) and given water throughout the experimental period. The temperature of the animal room was maintained at 27° ± 1° and environmental disturbances were reduced to a minimum. Phenylbutazone ("Butazolidin", Geigy) without added lignocaine was injected intramuscularly into the different groups in a dose range of 12·5–200 mg./kg. 48 and 24 hours before killing the rats. Thalidomide, 100 mg./kg., was administered orally one hour before the first injection of phenylbutazone and repeated 12 hourly during the experimental period. The rats were then killed for pathological examination.

Results

Stress Ulcers

In the control rats ulcers developed in the glandular part of the stomach, beginning as small petechial haemorrhages followed by erosion to the submucosal regions to form typical ulcers. The degree of ulceration was greater when the rats were isolated in individual cages than when they were grouped together. Treatment with thalidomide prevented the ulceration and this was associated with an absence of the hyperacidity which occurred in the control groups (Fig. 1). Treatment with thalidomide after establishment of ulcers had a curative action, the degree of ulceration being much less after four days than in the control groups (Fig. 2).

Phenylbutazone Ulcers

Gastric damage was significantly more severe in rats which were kept singly in individual cages than when they were grouped 2 or 4 in a cage (Fig. 3) and the degree of damage increased in all groups with increasing doses of phenylbutazone. In thalidomide treated rats this difference did not occur, the gastric damage in isolated rats being reduced to the same level as in aggregated rats (Fig. 4). The results from all the thalidomide treated rats may therefore be combined and in Figure 5 these are compared with those from rats treated with phenylbutazone alone at the same time. These combined results show that thalidomide did not diminish the damage caused by small doses of phenylbutazone and at higher doses of phenylbutazone thalidomide had only a slight protective effect.

Discussion

The importance of nervous factors in the pathogenesis of gastric ulcers is shown by the fact that ulcers can be induced by stress alone, using

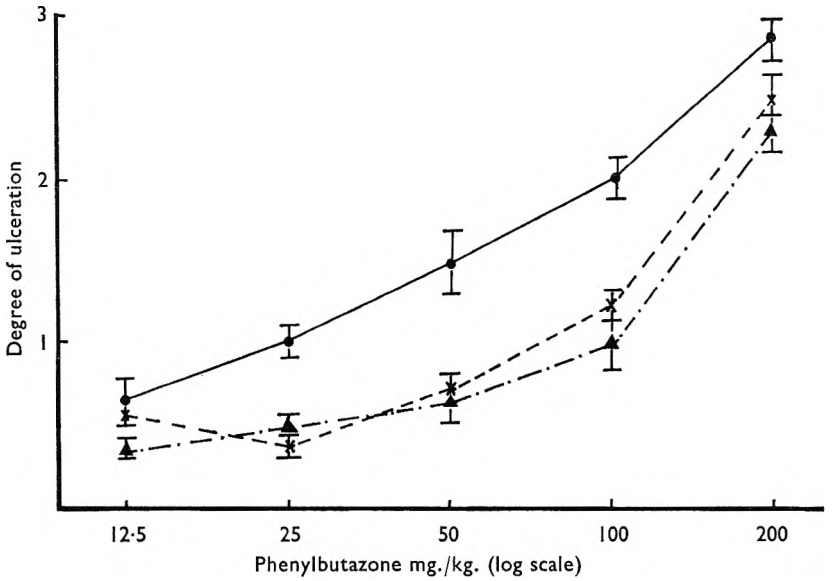

FIG. 3. Ulcerative action of phenylbutazone in grouped and single rats. Each point is the mean with standard error from four rats ●—● 1 per cage, X---X 2 per cage, ▲-·-▲ 4 per cage.

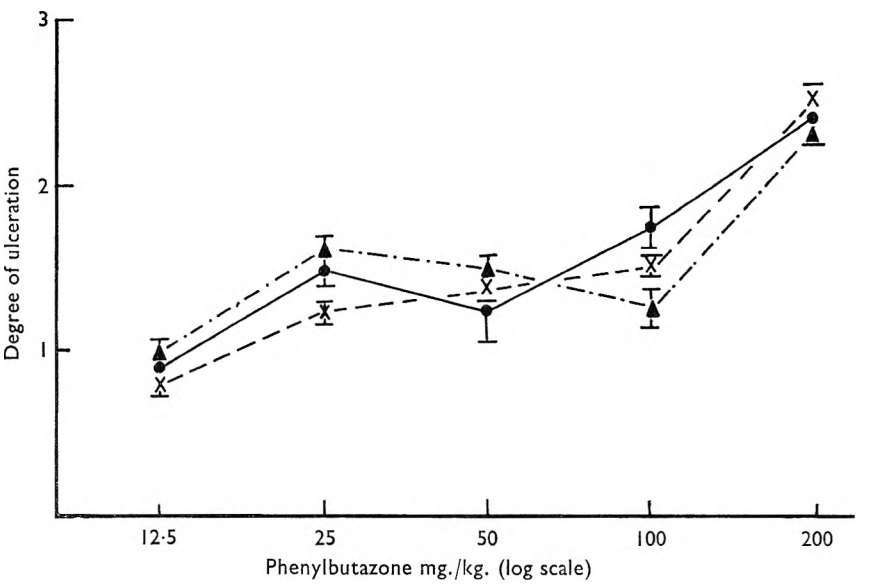

FIG. 4. Ulcerative action of phenylbutazone in grouped and single rats treated with thalidomide, 100 mg./kg. orally. Each point is the mean with standard error from four rats ●—● 1 per cage, X---X 2 per cage, ▲—▲ 4 per cage.

restriction in a Plaster of Paris bandage. However it is possible for stress factors to potentiate ulceration by other means, such as phenylbutazone. Experiments of this kind must be adequately controlled as has been emphasised by Bonfils and others[3]; also it is important carefully to standardise laboratory conditions and to avoid variations in the environment. Without these precautions fallacious results may be obtained[4].

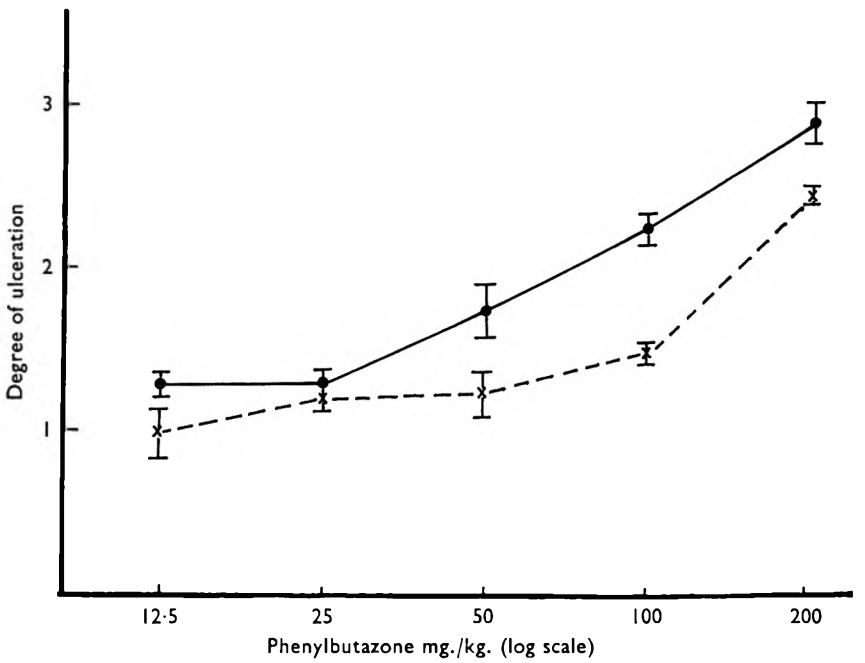

FIG. 5. Ulcerative action of phenylbutazone in normal and thalidomide treated rats. ●—● Phenylbutazone, X--X thalidomide and phenylbutazone.

Stress has been shown by Selye[5,6] to cause gastric ulcers through a central nervous mechanism. Thalidomide presumably prevented ulceration in stressed rats by inhibiting this central nervous mechanism which is responsible for the pathological changes occurring in the gastric mucosa. Preliminary observations which we have made in the guinea pig show that thalidomide in this species has an action against stress ulcers which is not explainable by a change in gastric acid secretion.

The ineffectiveness of thalidomide against phenylbutazone induced ulceration may be explained by the fact that the initial lesion in this type of ulcer is due to a specific local effect, chemical or endocrine, subsequently potentiated by gastric autodigestion[7]. However the different results in grouped and single rats suggest that a concomitant stress reaction may also be involved which is largely dependent on the environmental conditions. This may be inhibited by treatment with thalidomide. This nervous element may pass unrecognised, but by designing experiments such as we have described it is possible to separate the central nervous

mechanisms from other caustive factors involved in gastric ulceration.

The removal of this nervous factor by thalidomide in our experiments suggest that its anti-ulcerative action is mediated through the central nervous system.

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After Dr. Somers presented the paper there was a DISCUSSION.

AN ENZYMATIC METHOD FOR THE DETERMINATION OF PREDNISOLONE PHOSPHATE IN PHARMACEUTICAL PREPARATIONS

By P. F. G. Boon

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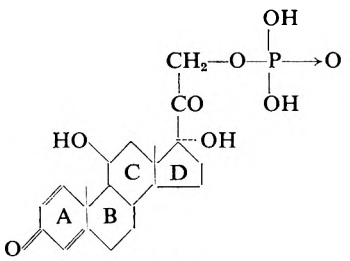
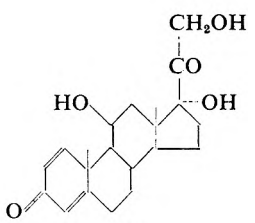
Received May 23, 1960

A method proposed for determining prednisolone phosphate in pharmaceutical preparations is based on enzymatic hydrolysis and ultra-violet spectroscopy; it is applicable to preparations that have undergone partial decomposition from prolonged or unsatisfactory storage.

The introduction of the 21-monophosphoric ester of prednisolone (I) in various pharmaceutical formulations has necessitated the development of a generally applicable analytical procedure, capable of distinguishing between the steroid ester and its decomposition products. Decomposition of prednisolone phosphate may occur by either of two mechanisms. The conjugated system in ring A is photolabile[1], undergoing drastic modifications of rings A and B; this change is accompanied by a reduction in ultra-violet absorption. The 21-phosphate ester linkage is susceptible to hydrolysis, liberating the relatively insoluble prednisolone (II), which further is degraded to the 17-aetio-acid (III) and 17-ketone (IV)[2–4]. Preparations undergoing accelerated storage tests may therefore contain (II), (III) and (IV), in addition to products of the photochemical reaction.



(I)



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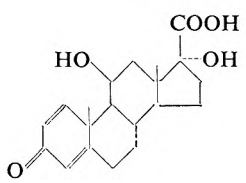

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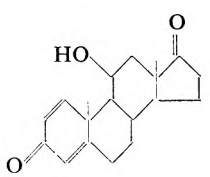

(IV)

No specific analytical methods for phosphoric esters appear to have been reported. A recent colorimetric procedure for the determination of steroid esters[5], based on hydroxamic acid formation, is not applicable to phosphoric esters. Other methods for the steroid alcohols depend on either the dihydroxyacetone side chain or the conjugated system in ring A,

both of which are closely associated with physiological activity. Methods based on the side chain include a number of widely used colour reactions, such as reduction of tetrazolium salts[6], reaction with a phenylhydrazine reagent[7] (the Porter-Silber reaction) or determination of the formaldehyde[8] produced by periodate oxidation, none of them directly applicable to the esterified alcohol. The second group of methods, based on the structure of ring A, includes ultra-violet absorption procedures or requires colour formation with sulphuric acid; these methods are applicable to simple aqueous solutions of the steroid ester but, without modification, frequently fail with the more complex preparations, especially if partial decomposition has taken place.

Ideally an analytical procedure should incorporate a measure of both functional groups and give no response to decomposition products. In one possible approach, prednisolone and its degradation products are extracted from the aqueous sample by a suitable immiscible solvent and the residual water-soluble steroid ester is then determined by ultra-violet spectroscopy. This procedure requires a fore-knowledge of all degradation products and is limited in application to formulations containing no ultra-violet absorbing components that are not extracted by the selected solvent. A more promising approach involves measurement of either the steroid alcohol or the inorganic phosphate produced by deliberate hydrolysis. Determination of the steroid alcohol by ultra-violet spectroscopy after isolation by solvent extraction would be particularly useful, since the complete determination would then depend on both physiologically functional groups.

Hydrolysis of prednisolone phosphate by chemical means necessitates severe treatment and causes much degradation of the liberated alcohol. Consideration was therefore given to the possibility of hydrolysing under mild conditions using a suitable enzyme, of which several are commercially available. An enzymatic method has the particular advantage that interfering substances can, if necessary, be removed by extraction before hydrolysis, under the same conditions as are subsequently used for extraction of the hydrolytic product. High specificity is thus assured. However, since enzymatically catalysed reactions are reversible, they seldom reach completion unless one of the hydrolytic products is removed continuously; further, enzyme poisons must be absent, or at least present in quantities insufficient significantly to alter the reaction rate. These and other matters bearing on the hydrolysis of prednisolone phosphate were therefore studied more closely.

Experimental

An "alkaline intestinal phosphatase" and a "wheat-germ acid phosphatase" were obtained from a commercial source. Hydrolyses were carried out by incubating mixtures of standard prednisolone phosphate solution, buffer and enzyme solution for different times. The extent of hydrolysis was determined after isolating the liberated alcohol by extraction with ether or chloroform, evaporation to dryness, solution of the residue in ethanol and ultra-violet spectroscopy. The ultra-violet

absorption of prednisolone disodium phosphate in water shows a maximum at 247 mμ, E(1 per cent, 1 cm.) 312.

Variables likely to influence hydrolysis are pH, temperature and the substrate : enzyme concentration ratio. Preliminary experiments showed the wheat-germ phosphatase to have a much slower reaction rate and a less favourable equilibrium position than the other enzyme. It was therefore discarded, and the alkaline phosphatase was used for all subsequent experiments. This had diminished activity below pH 8·0; above pH 9·0 loss of liberated alcohol by degradation of the dihydroxyacetone structure became significant. Moreover, the reaction was retarded by ionisable phosphate, which may be present in the sample as buffer. This interference was eliminated by precipitation of phosphate with calcium chloride, but to obtain adequate removal of phosphate a pH of not less than 8·6 was required. The hydrolysis rate did not vary significantly over the temperature range 25° to 50°. The speed of reaction and its completion were facilitated by high enzyme or low substrate concentrations or both together. The liberated alcohol may be extracted by ether or chloroform, the latter being preferred because of its greater efficiency. Prolonged or unsatisfactory storage may lead to the formation of the steroid alcohol and its decomposition products which may be removed by a preliminary solvent extraction.

Method

Reagents

Glycine buffer. Dissolve 7·505 g. of glycine and 5·85 g. of sodium chloride in sufficient water to produce 500 ml. Adjust to pH 8·6 with 0·2N sodium hydroxide. *Calcium-magnesium solution.* Dissolve 1 g. of calcium chloride and 1 g. magnesium chloride in sufficient glycine buffer to produce 20 ml. *Purified alkaline phosphatase solution.* Dissolve sufficient alkaline phosphatase in 20 ml. water to produce an enzyme concentration of 1,000 units per ml. Shake gently for 1 minute with an equal volume of chloroform. After separation discard the chloroform.

Procedure

Transfer an aliquot of the sample containing 2·5 mg. of prednisolone phosphate to a 250 ml. glass-stoppered separator, dilute to 15 ml. with water, add 25 ml. of chloroform, stopper, shake for 1 minute, and set aside for 2 minutes. Transfer the lower chloroform layer to a second separator, and wash it with 2 ml. of water. Reject the chloroform, and transfer the washings to the first separator. Repeat the extraction and washings with a second 25 ml. portion of chloroform. Transfer the aqueous phase and washings to a 25 ml. volumetric flask, dilute to volume with water, and mix. Transfer a 5.0 ml. aliquot to a glass-stoppered boiling tube. Add 5 ml. of *glycine buffer*, 1 ml. of *calcium-magnesium solution*, 1 ml. of *purified alkaline phosphatase solution* and mix by swirling. Stopper the tube, bring to 37° by immersion in warm water, and then maintain at 37° for 4 hours. Cool to room temperature. Transfer the contents of the tube to a 250 ml. stoppered separator with the aid of two

2 ml. portions of water. Add 25 ml. of chloroform to the separator by the tube. Stopper, shake for 1 minute, and set aside for 2 minutes. Transfer the lower chloroform layer to a second separator, and extract the aqueous phase with two further 25 ml. portions of chloroform. Combine the chloroform extracts, wash with 2 ml. of water, and transfer quantitatively to a 250 ml. glass-stoppered flask. Distil to near dryness on a steam bath, removing the last 1 or 2 ml. of chloroform in a current of air. Cool to room temperature. Add 25·0 ml. of ethanol, stopper, and swirl to dissolve the residue.

Determine the extinction of this solution against a blank of ethanol in 1 cm. cells at λ max near 242 mμ. Calculate the prednisolone phosphate

TABLE I

REPLICATE DETERMINATIONS

Determination	Prednisolone phosphate per cent w/v	
No.	Lotion	Injection
1	0·267	2·05
2	0·272	2·08
3	0·275	2·02
4	0·278	2·02
Mean	0·273	2·04
Range	0·267–0·278	2·02–2·08

content of the sample, taking the E(1 per cent, 1 cm.) value of prednisolone alcohol as 415 and 1·344 as the conversion factor for alcohol to ester disodium salt.

RESULTS

Recovery experiments were made on aqueous solutions of a recrystallised specimen of the steroid phosphate disodium salt. This derivative is hygroscopic hence solutions of approximately known strength were prepared and their equivalent steroid alcohol contents determined from the ultra-violet absorption of direct aqueous dilutions. The alcohol recovered after hydrolysis by the proposed method was calculated as a percentage of the figure obtained by direct dilution. The mean of 10 such determinations was 97·9 per cent and the standard deviation 1·0 per cent. The precision attainable is shown (Table I) by replicate determinations on a production lotion and injection expected to contain 0·27 and 2·0 per cent w/v of active agent respectively.

The proposed method has been applied to preparations for which the direct solvent-extraction method fails. The prednisolone phosphate content of fresh preparations containing *p*-hydroxybenzoic acid esters as preservative may be determined by the rapid solvent-extraction method. However, this procedure is rendered inapplicable by decomposition of the preservative on prolonged storage, resulting in formation of a non-extractable ultra-violet absorbing substance. The proposed enzymatic procedure, by virtue of its "before and after" extractions, achieves a

complete separation of steroid from both the preservative and its decomposition products.

Experimental preparations containing monothioglycerol gave unexpectedly low results. Initially this was thought to be due to enzyme poisoning by the thiol group; however, a four-fold increase in enzyme concentration produced the same result, and subsequent work indicated chemical interaction with the prednisolone phosphate. The proposed method failed on application to an experimental injection product containing nicotinamide, which is only slowly extracted from aqueous solutions by the usual solvents. The slow rate of extraction made it impracticable to attempt a complete preliminary extraction, but the amounts extracted after hydrolysis were sufficient to invalidate the ultra-violet procedure. This difficulty was overcome by applying the tetrazolium reduction colour reaction to the

TABLE II

RESULTS OF ANALYTICALLY PREPARED SAMPLES

	Preparation	Prepared strength per cent	Strength found per cent
1.	Enema containing *p*-hydroxybenzoates	0·033 w/v	*0·033 w/v
2.	Suppository	0·50 w/w	0·49 w/w
3.	Lotion containing neomycin and nicotinamide ..	0·26 w/w	**0·25 w/w
4.	Injection containing nicotinamide	3·3 w/v	**3·3 w/v
5.	Injection containing phenol and sodium formaldehyde sulphoxylate	2·9 w/v	2·9 w/v

* In this determination the initial extraction was acidified.

** In these determinations final measurement was made by the tetrazolium reduction colour reaction.

extracted alcohol. Experimental results are summarised in Table II. No enzyme poisons have been encountered, the method has been successfully applied to preparations containing phenol, *p*-hydroxybenzoic esters, phenylethanol, merthiolate and neomycin. Determinations giving unexpectedly low results, however, should be repeated with an increased enzyme concentration as a check against inhibition. The proposed method is also applicable to the 21-monophosphoric esters of hydrocortisone and dexamethasone.

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After Mr. Boon presented the paper there was a DISCUSSION.

THE PRECISION OF SOME PROCEDURES IN PHARMACEUTICAL ANALYSIS

PART II. TITRATIONS

BY A. R. ROGERS

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Received May 23, 1960

Estimates have been made of the variation of the results obtained by students in eight schools of pharmacy and by analysts in five industrial laboratories performing simple titrations. Coefficients of variation in the range 0·1 to 0·2 per cent were typical of the industrial analysts. The corresponding figures for the students were mostly in the range 0·2 to 0·5 per cent.

IN Part I[1] of this series, estimates were made of the magnitude of the variance of the volumes delivered by 10- and 20-ml. pipettes in the hands of a number of students. The total figure was the sum of the between-students variance and the between-pipettes variance. Similar estimates were made of the magnitude of the variance of the volumes delivered by burettes. Again the total figure was the sum of contributions from the students (errors of reading) and the apparatus (errors of calibration). The between-pipettes and between-burettes variances found in Part I can be only an approximate guide to the variances of other batches of volumetric apparatus, but the between-students variances are probably good estimates of between-analysts variances for these operations.

In the present paper, the total variation associated with the use of a pipette and a burette has been compared with the variation of titres found when students and industrial analysts take by pipette an aliquot portion of a sample and titrate it with reagent added from a burette. The objectives were to discover whether the reproducibility of results obtained by students is similar to that of results obtained by industrial analysts and if so, at any rate for some titrations, whether the variation is significantly greater than that which can be attributed to variation associated with calibration and correct usage of the apparatus. A high variation could be attributed in part to difficulty in detecting the end point.

There are comparatively few reports of the precision with which volumetric analysis is normally carried out on a routine basis, as in testing for compliance with pharmacopoeial specifications. Of these, some relate to titrations which involve weighings or to back-titrations, and so are not directly relevant to the subject of this paper. Bishop[2] has stated that the accuracy and precision of routine volumetry and gravimetry are about the same, roughly 0·1 to 0·5 per cent.

Some figures relating to titrations made by large numbers of students have been published. Students of Farquhar and Ray[3] beginning their laboratory course in chemistry gave results which indicated a coefficient of variation, that is, a relative standard deviation, of about 3 per cent for the assay of a sample of vinegar with the use of standard 0·25N acid and approximately 0·25N alkali. Cooper[4] published results obtained by

students in their first semester of quantitative analysis, who achieved a coefficient of variation of about 0·9 per cent for the assay of samples of sodium hypochlorite solution. Chapman[5] found that beginning students were able neither to obtain closely agreeing results in volumetric analysis nor to obtain close agreement with the results obtained by other students; coefficients of variation of about 0·5 per cent were reported for simple acid-base titrations. Park[6] reported that beginning students in quantitative analysis obtained a coefficient of variation of about 0·3 per cent for the volumetric determination of chloride by the Mohr and the Fajans methods.

Results Obtained by Brighton Students

For several years, detailed records have been kept of all classwork in practical quantitative analysis made by first-year degree and diploma students of the Brighton School of Pharmacy. Some results obtained

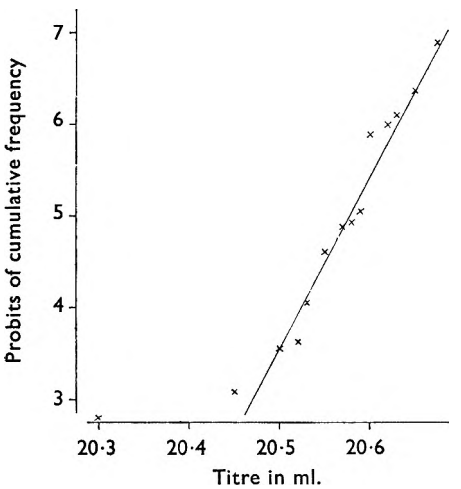

Fig. 1. Distribution of 70 results for the titration of 20 ml. of 0·05M sodium edetate with 0·05M lead nitrate.

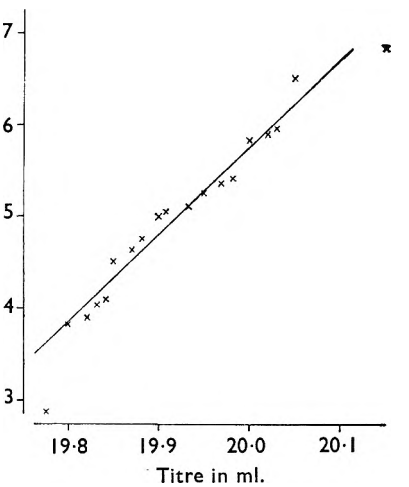

Fig. 2. Distribution of 58 results for the titration of 20 ml. of 0·1N iodine with 0·1N sodium thiosulphate.

by second-year students were also available; they showed no improvement over those obtained by first-year students. The figures were taken directly from the students' original laboratory notebooks, and the nature of the supervision was such that all results obtained by the students were recorded and not merely the "two best" or any other selection. All calculations, including subtractions of burette readings, were checked.

The figures used were the titres obtained when each of a large number of students (30 or 40) took by pipette an aliquot portion of a given solution and titrated it with a given volumetric solution run in from a burette. Each student used his own pipette and burette. The titrations were made in duplicate, although occasionally a student would record the result of three or four replicate titrations, and occasionally only a single result would be available.

Variances were calculated from standard deviations estimated from the reciprocal of the slope of a plot of probit of cumulative frequency against titre[1,7]. Note that a plot on ordinary graph paper of probit of cumulative frequency against titre is equivalent to a plot on probability paper of cumulative frequency against titre. When the plot was slightly curved, a straight line was drawn by eye through those points that came within the range of probits four to six, although attention was also paid to the trend of points outside this range. When the plot was highly curved or S-shaped, the results were discarded; over one-third of the Brighton results were discarded for this reason.

A typical graph is shown in Figure 1, which is derived from the 70 results obtained by 35 students titrating 20 ml. of 0·05M sodium edetate with 0·05M lead nitrate with xylenol orange as indicator. (I am grateful

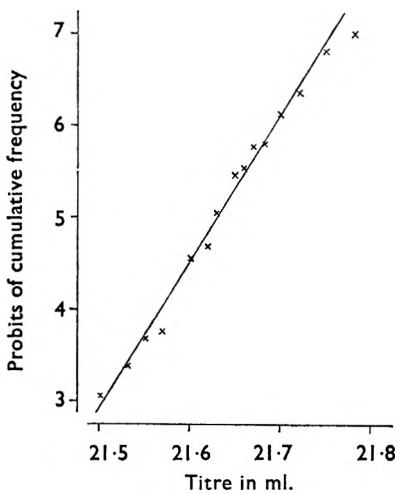

FIG. 3. Distribution of 76 results for the titration of 20 ml. of 0·5N hydrochloric acid with 0·5N sodium hydroxide.

to Mr. C. A. Johnson for suggesting this titration which has a very sharp end point.) The standard deviation calculated from the graph is 0·056 ml., which is equivalent to a coefficient of variation of 0·27 per cent; the coefficient of variation calculated directly from the 70 titres by summing squares of deviations, and so on, is 0·36 per cent. The graphical method of estimation is preferred because it gives less weight to the "outliers" (results a long way from the mean) which probably arise from mistakes rather than from an accumulation of small normally distributed chance errors.

Figure 2 shows a graph which is a good approximation to a straight line, but the coefficient of variation of 0·51 per cent seems high for such a simple titration, namely, the titration of 20 ml. of 0·1N iodine with 0·1N sodium thiosulphate. By contrast, Figure 3 shows a graph constructed from results for the titration of 20 ml. of 0·5N hydrochloric acid with 0·5N sodium hydroxide with methyl orange as indicator; the coefficient of

variation is 0·28 per cent. Over 30 graphs of this type are now available based on the results of Brighton students.

It is customary in the schools of pharmacy to require a student to make every titration in duplicate, and to hand in two separate results. This procedure sometimes has the advantage of showing a student that it is possible to get close agreement between replicate determinations, and it also provides additional practice in the various exercises. On the other hand, close agreement of replicates may blind a student to the possibility of bias in the procedure or in the way in which it has been carried out. An analyst in an industrial control laboratory will normally perform a

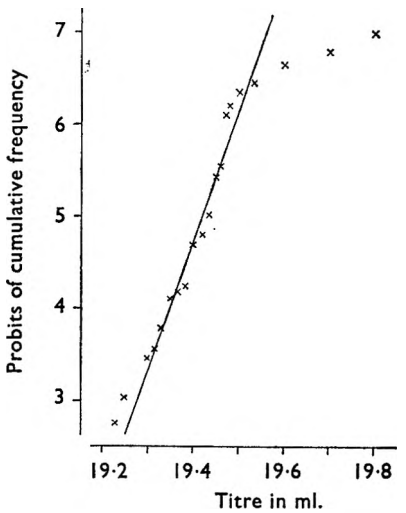

FIG. 4*a*. Distribution of 80 results for the titration of 20 ml. of 0·1N silver nitrate with 0·1N ammonium thiocyanate. Each student made two titrations.

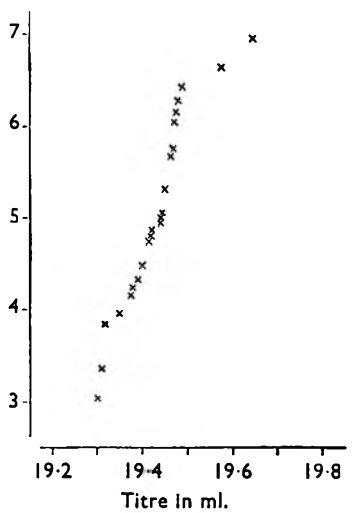

FIG. 4*b*. Distribution of 40 mean results obtained as in *a*.

single determination, and replicate analyses will be done only in special circumstances, and then preferably by another analyst on another occasion. A replicate titration made concurrently with or immediately after a first determination by the same analyst in the same laboratory adds little to the accuracy of the result. It may provide a check against a gross error or mistake, and, if the two duplicate results differ, show that further titrations are necessary[8,9].

The variation within students will almost always be less than the variation between students. The coefficient of variation calculated from all of the results will therefore probably be an underestimate of the variation between students and it might seem best to use the students' mean results as the basis of calculations. Although it is always possible to form an estimate of the coefficient of variation from the means of the students' results, the departure from a normal Gaussian distribution is then usually greater than when all the individual results are used, and so the estimate

may be very inaccurate. An example of this is illustrated in Figure 4. Figure 4*a* shows a graph constructed from the 80 results obtained by 40 students, each of whom made in duplicate the titration of 20 ml. of 0·1N silver nitrate with 0·1N ammonium thiocyanate with ferric ammonium sulphate as indicator. The line is reasonably straight if the three highest figures (19·6, 19·7 and 19·8 ml.) are ignored. The coefficient of variation calculated from the slope of the line is 0·36 per cent. Figure 4*b* shows the graph constructed from the 40 mean values, and even if a few "outliers" are ignored, it is still not possible to draw a straight line of good fit and the coefficient of variation cannot be estimated at all accurately.

Unless otherwise stated, all coefficients of variation quoted in this paper have been calculated from the individual results, despite the limitations of this procedure.

The coefficient of variation of results found for a given type of titration varied considerably from one group of students to another, and a value as low as 0·2 per cent could be regarded as unusually good. For the easier titrations, a coefficient of variation of 0·3 per cent was typical, and for the more difficult about 0·5 per cent. Of a total of 34 estimated coefficients of variation, distributed between 13 types of titration, five were below 0·3 per cent, eight were between 0·3 and 0·4 per cent, eight were between 0·4 and 0·5 per cent and 13 were above 0·5 per cent. Another 28 sets of results were discarded because of marked curvature of the probit-titre graphs.

An example of the difference between one group of students and another is the following set of coefficients of variation for the titration of 10 ml. of 0·6 per cent hydrogen peroxide solution with 0·1N potassium permanganate, obtained by seven groups of students: 0·27, 0·28, 0·23, 0·61, 0·44 and 0·19 per cent.

It is likely that these widely differing results reflect differing abilities of different groups of students to adhere to established and approved manipulative techniques. Observations in class and during examinations showed that even after 2 years of training many students would still mis-use a pipette or a burette or would fail to add the correct reagents in the correct amounts. Gregorczych[10] has stated that the main sources of analytical errors are the disregarding of analytical instructions and carelessness in performing the work. This view is supported by preliminary study of the results of students performing gravimetric exercises or titrations in which the sample is taken by weight.

It has therefore not been found possible, as was hoped, to draw up a list of titrations and the corresponding estimates of coefficients of variation found with Brighton students.

Results Obtained by Students in Other Schools of Pharmacy

Titration results obtained by students in seven other schools of pharmacy were collected. (I am grateful to the lecturers concerned for their kind co-operation.) The general pattern of figures was similar to that found at Brighton. For example, the coefficients of variation of the results in the titration of 20 ml. of 0·6 per cent hydrogen peroxide solution with

0·1N potassium permanganate were 0·33, 0·36, 1·27 and 0·34 per cent, respectively, from four groups of students in three schools.

Of a total of 14 estimated coefficients of variation, distributed between eight different types of titration, three were below 0·3 per cent, six were between 0·3 and 0·4 per cent and five were above 0·5 per cent. Another eight sets of results had to be discarded, because of marked curvature of the probit-titre graphs. The group size of students ranged from 10 to 38; usually each student provided two results.

Results Obtained by Analysts in Industrial Laboratories

Titration results obtained by analysts in the control laboratories of five pharmaceutical manufacturers were collected. (I am grateful to the Chief Analysts concerned for their kind co-operation.) Eight sets of figures were obtained, of which one set had to be discarded because the distribution was far from normal. The seven remaining sets were either acid-base, (*a*) to (*f*), or chloride-silver, (*g*), titrations in which 25 ml. of solution was taken by pipette and titrated with reagent. The coefficients of variation were estimated as (*a*) 0·13 per cent, (*b*) 0·15 per cent, (*c*) 0·09 per cent, (*d*) 0·14 per cent, (*e*) 0·26 per cent, (*f*) 0·09 per cent and (*g*) 0·18 per cent. The numbers of analysts per group were (*a*) 38, (*b*) 7, (*c*) 10, (*d*) 10, (*e*) 4, (*f*) 5 and (*g*) 19. Each analyst provided two results, except that each analyst in (*g*) provided one result only.

Because of the small group sizes and the close agreement of the results, it was not certain that use of the probit-titre graph was always the best way to estimate the coefficient of variation, and so direct estimates were also made in the usual way by summing squares of deviations, and so on. As already stated, this direct method may overestimate the amount of variation. The direct estimates were (*a*) 0·19 per cent, (*b*) 0·17 per cent, (*c*) 0·10 per cent, (*d*) 0·13 per cent, (*e*) 0·27 per cent, (*f*) 0·09 per cent and (*g*) 0·23 per cent.

Discussion

It is clear that the hope expressed by Saunders and Fleming[11], that the data for calculating the percentage standard deviation of the different assay methods of the B.P. is available in the schools of pharmacy, cannot be fulfilled. The coefficients of variation of the students' results are often greater than those of the industrial analysts by a factor of two or three, and there is little agreement from one school to another or even from one group of students to another group within the same school.

If the figures obtained for calibration and use of pipettes and burettes and reported in Part I[1] are accepted, then a coefficient of variation within the range 0·12 to 0·20 per cent might be expected in a titration with a sharp end point where 25 ml. of solution taken by pipette gives a titre of 25 ml. of a colourless reagent, according to whether one or many pipettes and burettes are used. The industrial results mostly fall within this range, and so the estimates of Part I are thought to be reasonably accurate.

It seems unlikely that much useful information on the precision of such procedures in pharmaceutical analysis as titrations involving weighings

and gravimetric assays can be obtained from the results of students' work, although very large quantities of data are available. Some useful results might come from the work of individual experienced teachers of pharmaceutical analysis, but the main hope lies with the industrial laboratories. By the fairly frequent but irregular submission of suitably disguised "standard" test samples to some or all of the analysts in a laboratory, estimates can be made of the within-analysts and between-analysts variances, and the performance of each individual can be checked[12–14].

Acknowledgements. I thank Mr. D. C. M. Adamson (Glaxo Laboratories Ltd.), Dr. G. E. Foster (Burroughs Wellcome & Co. Ltd.), Dr. D. C. Garratt (Boots Pure Drug Co. Ltd.), Dr. R. E. Stuckey (The British Drug Houses Ltd.) and Mr. W. Smith (Allen and Hanburys Ltd.); and Dr. L. M. Atherden (Bristol School of Pharmacy), Mr. P. H. Bailey (Leicester), Mr. D. R. Bragg (Sunderland), Mr. C. G. Butler (Bradford), Dr. R. Fleming (University of London), Dr. G. Kirk (Chelsea), and Dr. F. Perks (Portsmouth); and the hundreds of students and analysts who provided the data. I thank also the Council of the Pharmaceutical Society for the loan of a Monroe model CAA 10-3S electric calculating machine.

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After Mr. Rogers presented the paper there was a DISCUSSION.

PHYSICO-CHEMICAL STUDIES OF (1-METHYL-2-PYRROLIDYL) METHYL BENZILATE METHYL METHOSULPHATE

PART I. THE DETERMINATION OF (1-METHYL-2-PYRROLIDYL)METHYL BENZILATE METHYL METHOSULPHATE IN THE PRESENCE OF ITS BREAKDOWN PRODUCTS

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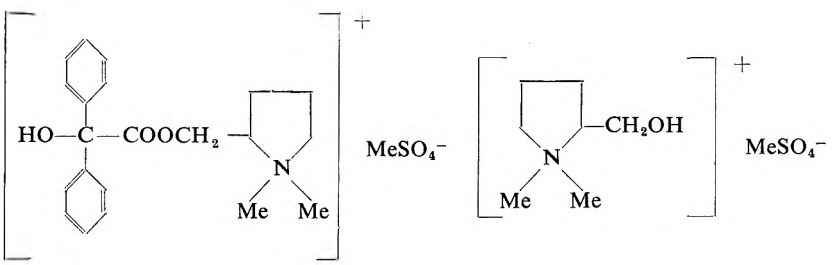
Received May 23, 1960

The determination of (1-methyl-2-pyrrolidyl)methyl benzilate methyl methosulphate by the formation of a chloroform-soluble, blue complex with ammonium cobaltothiocyanate is described. The optical density in chloroform at 322 mμ is directly related to the amount of compound present. The method is applicable in the presence of breakdown products and has been used to determine the concentration of (methyl-2-pyrrolidyl)methyl benzilate methyl methosulphate in tablets and in linctuses.

THE ester (1-methyl-2-pyrrolidyl)methyl benzilate methyl methosulphate (I) (poldine methosulphate, Nacton) can hydrolyse to give benzilic acid and *N*-methyl prolinol methyl methosulphate (II). The ultra-violet



I II

absorption spectrum of poldine methosulphate is almost entirely due to the benzilic acid moiety. Attempts to obtain an analytical separation from mixtures with benzilic acid failed. Bromothymol blue[1,2] and Orange II[3] have been used for the extraction and estimation of quaternary ammonium compounds with long side chains. Both the compound and the prolinol moiety react with these reagents.

Brown and Hayes[4] state that cetyltrimethylammonium bromide, but not phenyltrimethylammonium chloride give the reaction with ammonium cobaltothiocyanate described by Gnamm[5], and developed by van der Hoeve[6] and Wurzschmitt[7]. Helgren, Theivast and Campbell[2] used this reaction to determine the quaternary anti-acetylcholine substance "Tral."

It was found that poldine methosulphate but not *N*-methyl prolinol methyl methosulphate reacted with ammonium cobaltothiocyanate.

Experimental

The work of Brown and Hayes[4] is concerned with the determination of polyethylene glycol (PEG) mono-oleates. To 20 ml. of ammonium cobaltothiocyanate reagent is added 5 ml. of a solution of polyethylene glycol mono-oleate. After reaction is complete, the blue complex formed is extracted into chloroform and the optical density read at 318·5 mμ or

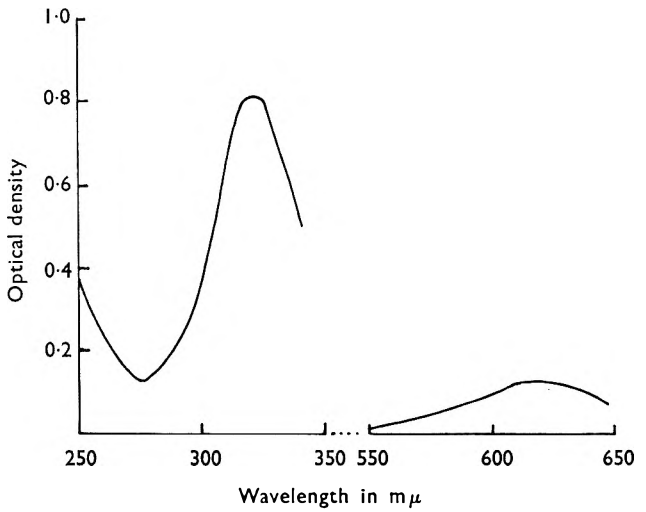


Fig. 1. Absorption curve of poldine methosulphate cobaltothiocyanate complex in chloroform.

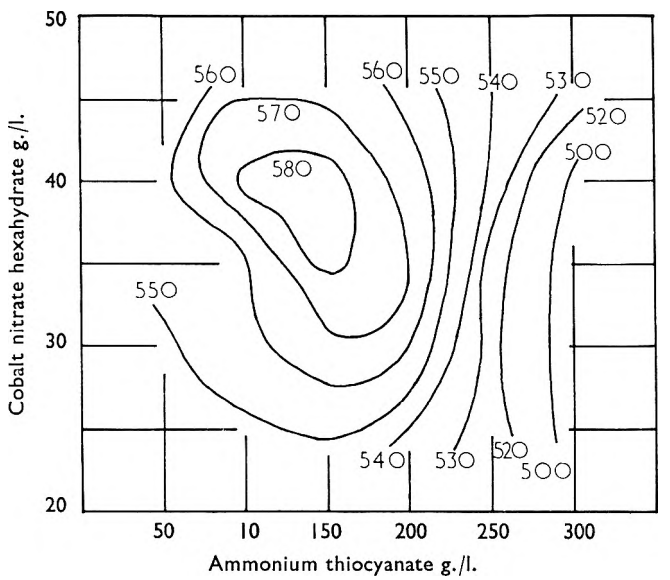


Fig. 2. Contour maps showing optimum cobaltothiocyanate reagent. Contours are optical density × 1,000 for poldine methosulphate.

620 mμ. Under carefully controlled conditions exact determinations could be made by this method.

When we applied this to a specially purified sample of poldine methosulphate prepared by the organic department of the Research Division of Beecham Research Laboratories Ltd., two differences were found. The absorption maxima were at 322 mμ and 620 mμ (Fig. 1). The optimum reagent composition was not the same.

TABLE I

DAY-TO-DAY VARIATIONS IN CALIBRATION CURVES FOR POLDINE METHOSULPHATE

Poldine methosulphate mg.	Optical density at 322 mμ, 1 cm. cell				
	A	B	C	D	E
0·3	0·103	0·105	0·105	0·103	0·104
0·6	0·204	0·227	0·201	0·212	0·225
0·9	0·330	—	0·325	0·332	0·318
1·2	0·451	0·454	0·459	0·459	0·464
1·5	0·574	0·587	0·580	0·579	0·586
Slope of regression line	0·396	0·397	0·403	0·400	0·405
Intercept of regression line	−0·024	−0·014	−0·029	−0·023	−0·026
Value calculated for 1·5 mg. compound 499	0·570	0·581	0·576	0·577	0·582

To determine the optimum reagent composition a series of reagents in which the concentration of cobalt nitrate hexahydrate was varied from 25 to 45 g./l. and ammonium thiocyanate from 50 to 350 g./l. was prepared. To 20 ml. of reagent was added 5·0 ml. of a solution containing 0·3 g./ml. poldine methosulphate. After reaction, the complex was extracted with chloroform and the optical density of the chloroform solution determined at 322 mμ. A contour map (Fig. 2) relating optical

TABLE II

EFFECT OF BREAKDOWN PRODUCTS ON THE ASSAY OF POLDINE METHOSULPHATE

Poldine methosulphate mg.	*N*-methyl prolinol methyl methosulphate mg.	Benzilic acid mg.	Optical density 322 mμ, 1 cm. cell
1·0	—	—	0·381
1·0	1·0	—	0·380
1·0	2·0	—	0·380
1·0	5·0	—	0·380
1·0	—	1·0	0·378
1·0	—	2·0	0·382
1·0	—	5·0	0·382
1·0	5·0	5·0	0·381

density to concentrations of the two components of the reagent was constructed. This compares with that obtained by Brown and Hayes[4]. The contour map shows a peak rather than a plateau, and the differences in optical density are much less. Subsequent work has shown that the type of contour map obtained and the optimum reagent composition varies from compound to compound.

METHOD

Reagents. Ammonium cobaltothiocyanate solution. Dissolve 37·5 g. of Analar cobalt nitrate hexahydrate and 150 g. of Analar ammonium

thiocyanate in water and make up to 1 l. with distilled water. *Chloroform.* Analar, suitable for ultra-violet spectrophotometry.

Procedure. Transfer by pipette 20·0 ml. of ammonium cobaltothiocyanate reagent to a 100 ml. separating funnel. To this, add by pipette, 5·0 ml. of a solution containing 0 to 0·3 mg./ml. of poldine methosulphate. Shake the funnel vigorously for 1 minute and allow to stand for 5 minutes. Add 5 ml. chloroform, shake vigorously for 1 minute and allow to stand for 5 minutes. Swirl the funnel gently to mix the chloroform layer and run off the chloroform layer into a 25 ml. volumetric flask. Extract with a further three portions of 5 ml. of chloroform. Wash the outside of the

TABLE III

DETERMINATION OF POLDINE METHOSULPHATE IN STORED TABLETS

Batch No.	Age months	mg./tablet	
		Declared	Found
E2/134	4	1·0	0·95
	4	1·0	0·98
	4	1·0	0·94
E2/124	2	2·0	1·94
	4	2·0	1·96
E2/150	2	2·0	2·00
	4	2·0	1·96

TABLE IV

DETERMINATION OF POLDINE METHOSULPHATE IN LINCTUSES

Sample	Age	mg./ml.	
		Declared	Found
A	1 year	0·75	0·76
B	1 year	0·25	0·29
C	6 months	0·25	0·27
D	6 months	0·75	0·77
C	1 year	0·25	0·27
D	1 year	0·75	0·77

stem of the separating funnel with chloroform using these washings to make the combined chloroform extracts up to the mark. Shake to mix. Centrifuge the chloroform solution in a stoppered centrifuge tube for 5 minutes at 2,000 r.p.m. to remove water droplets. Measure the optical density of the chloroform solution against a chloroform blank at 322 mμ in 1 cm. cells.

Prepare a calibration curve using 5·0 ml. of solutions containing 0·06, 0·12, 0·18, 0·24, 0·30 mg./ml. of poldine methosulphate. From the calibration curve read off the amount of compound in the original 5 ml. aliquot. An approximate result may be calculated from the equation $y = 0{\cdot}4x - 0{\cdot}025$, where y is the observed optical density and x is the amount of compound in the original aliquot in mg. The equation holds over the range $x = 0{\cdot}3$ to 1·5.

RESULTS

Calibration curve. Table I shows calibration curves obtained on different days with different batches of reagent. The slope and intercept

of the regression line, and the calculated value for 1·5 mg. poldine methosulphate are also shown. The calibration curve is linear over the range 0·3 to 1·5 mg.

Blanks. Helgren, Theivagt and Campbell[2] reported large blanks at the shorter wavelength, using this reagent. Our experience was similar to Brown and Hayes in that negligible blanks were obtained.

Breakdown products. The assay procedure was carried out using 1·0 mg. of the compound with the addition of from 1 to 5 mg. of benzilic acid or *N*-methyl prolinol methyl methosulphate, or both (Table II). No significant difference in optical density between these determinations was found.

Tablets and linctuses. The method has been used to determine poldine methosulphate in stored tablets (Table III) and linctuses (Table IV).

Other substances. Ammonium cobaltothiocyanate did not react with the tertiary amines caffeine, quinine, codeine, ephedrine, atropine, or the unquaternised compound. But reacted with *N*-methylprolinyldiphenylmethyl ether. All these reactions were carried out at the 1·5 mg. level.

Discussion

The ammonium cobaltothiocyanate reagent provides a means of distinguishing between quaternary ammonium compounds of high and low molecular weight and in particular between the ester poldine methosulphate and its parent alcohol *N*-methyl prolinol methyl methosulphate. It has enabled us to demonstrate the stability of the compound in pharmaceutical preparations.

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After Mr. Singleton presented the paper there was a DISCUSSION.

SEPARATION AND ESTIMATION OF PURINE AND PYRIMIDINE BASES FROM A HEATED SUSPENSION OF *Micrococcus flavus*

By K. W. Gerritsma and H. P. Levius

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A method has been described for the chromatographic separation of a mixture of purine and pyrimidine bases. They are identified as single substances in solution by the ultra-violet light absorption at a small number of wavelengths. Checks on the purity of the recovered bases are also described and the method is applied to the separation and estimation of purine and pyrimidine bases from the supernatant fluid of a heated suspension of *Micrococcus flavus*.

Complete hydrolysis of nucleic acids (NA) yields a sugar component, phosphate and purine and pyrimidine bases. Pyrimidine derivatives found in nucleic acids are cytosine (RNA and DNA), uracil (RNA), thymine and 5-methylcytosine (DNA). A fifth pyrimidine, 5-hydroxymethylcytosine, replaces cytosine in certain strains of coliphage. Purine bases found in both types of nucleic acids are adenine and guanine. Work, particularly by Smith and Wyatt[1], has made it clear that the tetranucleotide hypothesis must be abandoned as nucleic acids vary widely in the molar proportions of bases according to the material of origin. It is known that in RNA the number of nucleotides carrying an amino group in the 6 position, adenine and cytosine, is equal to the number having a 6-oxo group, guanine and uracil. This symmetry is also found in DNA, thymine replacing uracil, but other regularities in composition are also present; (*a*) the molar sum of the purines is equivalent to the molar sum of the pyrimidines; (*b*) the molar ratio of adenine to thymine is 1; and (*c*) the molar ratio of guanine to cytosine + methylcytosine is 1. Smith and Wyatt have estimated the proportions of purine and pyrimidine in DNA from *E. coli* as follows, basing their calculations on molar proportions to a total of 4: adenine 0·90, guanine 0·98, cytosine 1·03 and thymine 1·09.

The terms RNA and DNA denote classes of compounds of varying composition. It is not known whether all molecules of RNA and DNA in an organism have the same composition, but the relative proportion of bases vary widely in nucleic acids from various sources, and even possibly in nucleic acids from the same source under varying environmental and metabolic conditions. Nucleic acids absorb ultra-violet light in the region of 260 mμ. This absorption is attributed to the conjugated double bond system of the purine and pyrimidine rings. There is no significant difference in the absorption of DNA and RNA. The ultra-violet absorption curves of the bases are illustrated in Figure 1. On hydrolysis the extinction coefficient of a nucleic acid increases significantly since the sum of the extinction of the constituent nucleotides is greater than the extinction of the polynucleotides.

Chromatographic methods have been of help in the separation of nucleic acid hydrolysis products[2–11]. The method of hydrolysis used, besides yielding quantitative cleavage of both RNA and DNA should not result in deamination of the bases. Marshak and Vogel[9] established that 12N perchloric acid cleaves nucleic acids quantitatively and permits determination of bases. After applying the usual one-dimensional chromatographic process, using some such solvent as n-butanol saturated with water, the position of the spot corresponding to the individual base

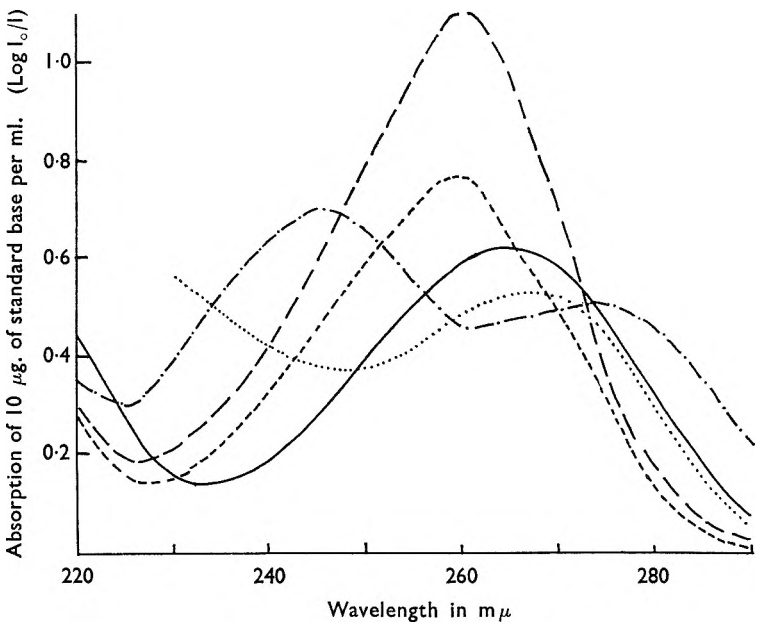

FIG. 1. Ultra-violet absorption curves for purine and pyrimidine bases at pH 7.
—— Thymine; – – – adenine; - - - uracil; –·–·– guanine; . . . . . cytosine.

or nucleotide is determined. The quickest and most useful method has been devised by Holiday and Johnson[12], in which the light transmitted by a Corning 9863 (230–400 mμ) filter shows the spots as dark patches against the background of paper fluorescence. The most suitable light source was found to be a low-pressure mercury resonance lamp. The spots are visible at concentrations of 0·5 μg./cm². Markham and Smith[2–5] have developed a method in which a permanent record may be kept of the chromatogram on photographic document paper exposed to ultra-violet light under suitable conditions. The print shows the absorbing substances as light areas on a black background. Other workers have developed a large number of methods and modifications for the isolation and identification of purines, pyrimidines and nucleosides[13–24]. These methods are complicated since they involve determinations of ultra-violet light absorption along a large range of wavelengths, and are made on mixtures of bases.

Experimental Methods

Purine and pyrimidine bases. The purity of commercial samples of cytosine, adenine, guanine, uracil and thymine was confirmed by spectrophotometric measurement of their solutions. Molecular extinction coefficients are given in Table I, together with figures obtained by other workers. Guanine showed 98·60 per cent of the theoretical weight of nitrogen by Kjeldahl determination.

Spectrophotometric measurements was by Unicam S.P.500 Ultra-violet spectrophotometer with 1 cm. matched silica cells.

Chromatographic methods. Whatman No. 1 papers were developed by the ascending technique for 24 to 30 hours at 19—20° after equilibration with n-butanol : water for 10 hours.

A Hanovia Chromatolite portable ultra-violet lamp fitted with suitable filters was used to examine the 260 mμ absorbing substances. The

TABLE I

Ultra-violet absorption data

Base	Extinction coefficient (ε)		Wavelength (mμ)	pH	Log I_0/I at 260 mμ (10 μg./ml. at neutral pH)
	Standard	Reference value*			
Uracil	8,512	8,600 (30)	258	7	0·73
Thymine ..	7,560	7,800 (30)	264	7	0·59
Cytosine ..	5,880	6,100 (11)	266	7	0·49
Adenine ..	12,725	13,000 (14)	260	0·1 N HCl	1·05
Guanine ..	10,180	11,000 (14)†	250	0·1 N HCl	0·465

* Figures in parentheses denote references in literature.
† Markham and Smith[3] and Wyatt[10] obtained a value of 10,500.

dark areas were marked in pencil outline and cut out. Blanks were prepared by tracing identical areas. Each spot and blank was eluted overnight in a test tube containing 4 ml. of water, and the absorption at 260 mμ measured spectrophotometrically. Five minutes was allowed to elapse with the sample in the cuvette, to allow fibres to settle.

The culture medium (Agar slopes). A commercially available dehydrated form of nutrient agar was used, prepared by Baltimore Biological Laboratories.

Organism. Micrococcus flavus MCI. NCIB 8134, obtained from the South African Bureau of Standards, Pretoria.

Preparation of the bacterial suspension. The slopes were innoculated and grown at 37° for one day, the organisms were washed off, suspended in distilled water, and then centrifuged at 2,000 *g* for 1 hour. After resuspension they were again centrifuged at 2,000 *g* for 1 hour. The supernatant liquid was again removed and the bacteria were resuspended in distilled water.

Preparation of the supernatant liquid of a heated suspension of M. flavus. The suspension previously described was heated for 1 hour in a water bath, the bacteria removed by centrifugation, and the supernatant was further heated to concentrate the solutes. This solution was evaporated *in vacuo*, and the solids obtained were hydrolysed by dissolving in 12N perchloric acid. This liquid was filled into neutral glass ampoules,

which were sealed and boiled for 1 hour. The acid was neutralised with ammonium hydroxide.

RESULTS

Ultra-violet Absorption of Standard Purine and Pyrimidine Bases in Neutral Solutions

Solutions of each of the five bases, adenine, guanine, cytosine, uracil and thymine, were prepared, containing 10 μg./ml. Absorption curves

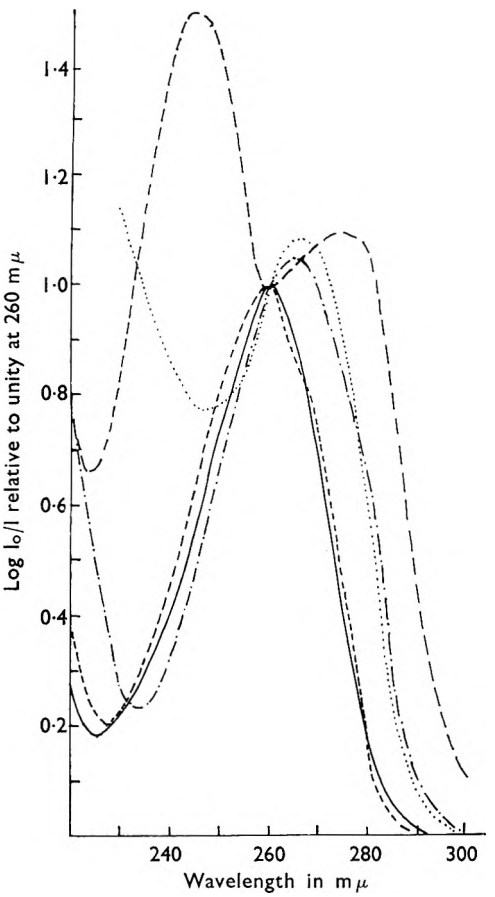

FIG. 2. Ultra-violet absorption curves for purine and pyrimidine bases at pH 7, derived from the corresponding curves on Figure 1.
—— Adenine; – – – guanine; - - - - uracil; –·–·– thymine; . . . . . cytosine.

are shown for each of these bases (Fig. 1). Guanine and adenine needed the addition of a small quantity of N NaOH to aid solution. This solution was then adjusted to neutrality with 0·1N HCl. Each of the other three solutions were also adjusted to pH 7, where necessary.

Identification of Purine and Pyrimidine Bases in Solution

A scheme, based on the results shown in Figure 1, was worked out which enabled the rapid identification of these bases in solution, when

present singly. Each result was recalculated relative to an optical density of unity at 260 mμ. The curves obtained are shown in Figure 2. From these curves, the ratios of the optical density at 240 mμ and 265 mμ to the optical density at 260 mμ were calculated. These ratios are denoted as N240/N260 and N265/N260 respectively.

TABLE II

ULTRA-VIOLET ABSORPTION OF PURINE AND PYRIMIDINE STANDARD BASES

Ratios of optical densities at various wavelengths

Base	$\frac{N240}{N260}$	$\frac{N265}{N260}$	$\frac{OH285}{OH270}$
Guanine ..	1·38	1·02	—
Cytosine ..	0·86	1·08	—
Uracil ..	0·44	0·88	1·13
Adenine ..	0·40	0·89	0·11
Thymine ..	0·32	1·05	—

N and OH refer to neutral solutions and to solutions containing 10 per cent N NaOH respectively. The figures denote the wavelength in mμ.

Hotchkiss[14] made use of pH absorption shifts to aid the determination of nucleic acid bases and a similar method is used here for uracil and adenine. Samples of these solutions were mixed with 10 per cent v/v of N NaOH and the absorption determined at 285 and 270 mμ. The ratio of the first reading to the second reading was calcultaed and was denoted OH285/OH270. The results are given in Table II. By the use of the values presented in this Table, it is possible to identify the base present in

TABLE III

SCHEME FOR THE IDENTIFICATION OF PURINE AND PYRIMIDINE BASES WHEN PRESENT AS SINGLE SUBSTANCES IN SOLUTION

Ratio	Result	Deduction
$\frac{N240}{N260}$	A Greater than 1·38	Guanine
	B Greater than 0·86, but smaller than 1·38	Cytosine
	C Smaller than 0·86	Uracil, adenine or thymine
$\frac{N265}{N260}$	D Greater than 0·95	Guanine, cytosine or thymine
	E Smaller than 0·95	Uracil or adenine
$\frac{OH285}{OH270}$	F Value greater than 1	Uracil
	G Value considerably less than 1	Adenine

a solution by neutralising the solution and measuring the absorption at 240, 260 and 265 mμ. The scheme adopted is represented in Table III.

Combination of results C and D will determine whether the base is thymine. From results A to E, all bases can be identified with the exception of uracil and adenine. If one or other of these bases are present, 10 per cent N NaOH is added and the absorption determined at 270 mμ and 285 mμ. If the ratio OH285/OH270 is greater than 1·0, the substance is uracil. If the ratio is smaller than 1·0, the base is adenine. Small variations in acidity may cause large deviations from the figures given. Where results are incompatible with those expected from R_F values then further experiments must be performed, by varying the pH

value of the sample. Results for the ratio N240/N260 from solutions recovered by chromatography sometimes showed a variation of as much as 0·3 higher than the theoretical. This discrepancy may be due to the presence of impurities with absorption at low wavelengths. Results for the ratio N265/N260 should agree to within 10 per cent.

As a confirmation to these tests, the relative R_F values of the spots are valuable[4,7,10]. R_F values have been found to vary considerably, thus they are not of value as absolute measurements. The variations encountered in R_F value could be accounted for by slight changes in temperature and solvent composition. The presence of acids or salts in

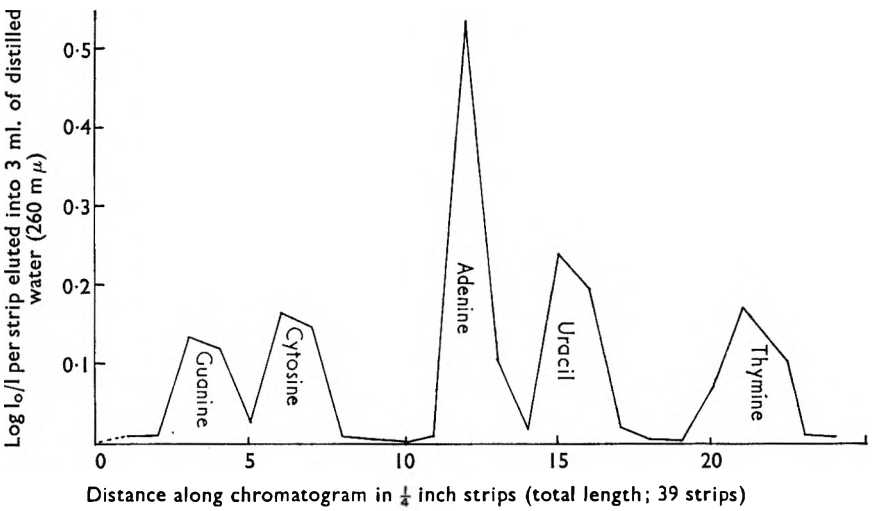

FIG. 3. Paper chromatogram of known bases. Sample contained 20 μg. of each of the five bases.

the solutions of bases, would also cause these variations. The pH of the solvent is known to have a marked effect on the movement of the bases. Increasing acidity causes purines to be held back more than pyrimidines. This effect is the apparent cause of the reversal of the position of adenine and cytosine noted by Wyatt[10] using an acidic solvent. Hotchkiss[14] using an alkaline system obtained a chromatogram in which the positions of uracil and adenine are in reverse to those obtained in the present paper.

Determination of Purity of Bases Recovered in Chromatography

The chromatographic techniques depend upon the complete separation of the bases. Readings below 240 mμ are unreliable in that the recovered bases yield high values; these may be derived from quantities of soluble extractive from the paper or the materials or from fluctuations in pH. Hotchkiss[14] has suggested that undue weight should not be placed upon comparisons made in this portion of the spectrum. Thus for practical purposes, it is better to rely on a small number of readings as described in the scheme for identification.

Separation and Estimation of Purine and Pyrimidine Bases from Solutions of Known Composition

A solution was made containing 0·1 per cent of each of the five bases. Spots were prepared on the chromatographic paper, from a short capillary tube of known capacity. The chromatogram was developed as described previously. By running the chromatogram for 24 to 30 hours it was possible to separate the spots completely. By applying the data given in Tables I–III, the amount of each base recovered from the mixture was calculated. Figure 3 shows a chromatogram that was obtained by cutting the chromatogram into strips. Table IV shows the quantitative results obtained in two experiments. Recoveries are quantitative, with

TABLE IV

THE SEPARATION, IDENTIFICATION AND ESTIMATION OF BASES

	1st expt.	2nd expt.	1st expt.	2nd expt.	1st expt.	2nd expt.	1st expt.	2nd expt.	1st expt.	2nd expt.
Rf value	0·05	0·08	0·13	0·15	0·26	0·29	0·36	0·38	0·54	0·54
N240/N260	1·44	—	1·07	—	0·69	—	0·65	—	0·56	—
N240/N260 for standard solution	1·38	—	0·86	—	0·40	—	0·44	—	0·32	—
N265/N260	1·01	—	1·09	—	0·91	—	0·90	—	1·03	—
N265/N260 for standard solution	1·02	—	1·08	—	0·89	—	0·88	—	1·05	—
OH285/OH270	—	—	—	—	0·16	—	1·08	—	—	—
OH285/OH270 for standard solution	—	—	—	—	0·11	—	1·13	—	—	—
Base present	Guanine		Cytosine		Adenine		Uracil		Thymine	
μg. of base (recovered)	32·85	17·30	19·57	19·95	19·89	18·45	36·75	18·65	37·17	18·00
μg. of base (actual)	38·95	18·86	19·55	19·70	19·73	19·34	39·01	19·38	39·00	19·14
Recovery (per cent)	84·5	91·8	100·0	101·2	100·1	95·5	94·2	96·3	95·5	94·2

an error less than 5 per cent. Guanine alone yields a poor recovery, possibly because its extinction coefficient has a critical relationship to the pH. It should be noted that guanine loses its maximum absorption in a short waveband (245 mμ) as the pH moves in the alkaline direction. On the other hand, it loses its maximum absorption in its long waveband (275 mμ) as the pH moves in the direction of acidity. The results obtained in this experiment show that guanine does not move appreciably in the solvent used, which is contrary to the results found by Hotchkiss[14].

Separation and Estimation of Purine and Pyrimidine Bases from the Supernatant of a Heated Suspension of M. flavus

A suspension of *M. flavus* was filled into neutral glass ampoules and sealed. Some were autoclaved at 115° for 30 minutes and the others stored at room temperature. After autoclaving, the heat-treated ampoules were stored at room temperature with the untreated ampoules. After approximately 24 hours, the supernatant fluids were examined spectrophotometrically (Fig. 4). The data for the purine and pyrimidine contents of the supernatant liquid are given in Table V.

After clarification of the supernatant liquid, the unautoclaved suspension exhibited the normal absorption. The autoclaved solutions showed greater absorption with a peak at 255 mμ.

Discussion

The supernatant solution from a heated suspension of *M. flavus* appears to have the peak of absorption at 255 mμ. We have found *Eschericia coli* and *Bacillus subtilis*, when treated in a similar manner, to have the peak absorption of the supernatant fluid at 260 mμ, corresponding to the absorption of nucleic acid. Others have reported

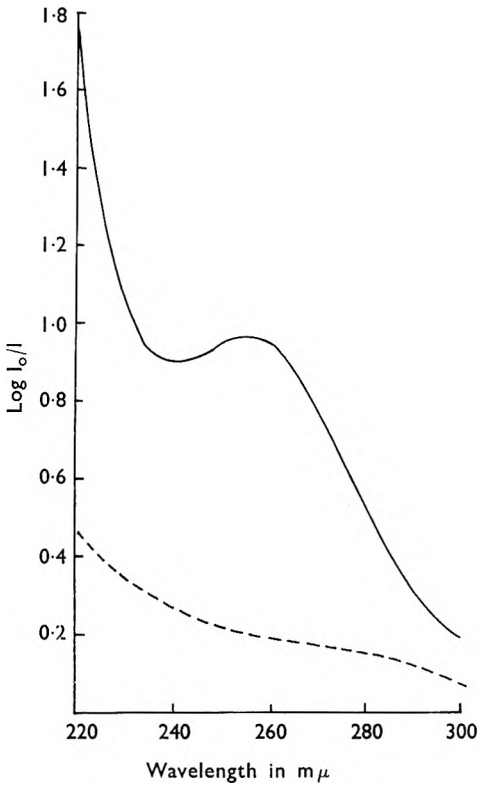

Fig. 4. Ultra-violet absorption of supernatant fluid from *M. flavus*.
- - - - Before autoclaving.
—— After autoclaving.
Bacterial concentration arbitrary.

the release of bacterial exudate under various conditions with maximum absorption at 260 mμ([25–28]). It appears that the locations of the absorption peaks of the supernatant solutions are dependent upon such factors as the age of the culture and the temperature at which they were stored. In this work, however, these factors remained constant.

In the supernatant of the organism examined, we find the sum of the cytosine and adenine to be equal to the sum of the guanine, uracil and thymine molecules, as did Elson and Chargaff[29]. *M. flavus* has been shown to have an exceptionally low thymine content and a high uracil and adenine content, which may account for the peak absorption at

255 mμ in the supernatant, rather than at the expected 260 mμ. The peak absorption of thymine solutions is at 265 mμ, thus in a mixture of the five bases, a paucity of this compound would yield a mean reading at a wavelength somewhat lower than normal. The low value obtained for thymine also indicates an unusually high predominance of RNA over DNA.

The purpose of the chromatographic study was not so much to investigate the mechanism of the release of cell exudate, as to attempt an insight into the distribution within the cell of nucleic acid. The results show

TABLE V

SEPARATION AND ESTIMATION OF PURINE AND PYRIMIDINE BASES FROM THE SUPERNATANT OF A HEATED SUSPENSION OF *M. flavus*

RF value	N265/N260	N240/N260	OH285/OH270	Base present	Log I_0/I*	μg. of base per spot	Molar ratio of bases
0·07	1·057	1·4	—	Guanine	0·087	7·49	5·0
0·17	1·01	1·09	—	Cytosine	0·128	10·45	9·4
0·27	0·78	0·86	0·29	Adenine	0·211	8·35	15·5
0·37	0·91	0·86	1·09	Uracil	0·399	21·90	19·5
0·47	1·00	0·86	—	Thymine	0·012	0·82	0·7

* Per spot eluted into 4 ml. of distilled water.

that most of the nucleic acid-containing tissues are involved in the release of cell exudate. This suggestion is prompted by the fact that the bases found in the supernatant are in the proportions expected when extracting DNA and RNA from intact cells. This postulation has a serious criticism, since it is based on the assumption that nucleic acids in different sites of a particular cell are not constant in their content of bases. Davidson[11] however, cites evidence obtained by several workers leading to the conclusion that the DNA complement of any one nucleus may be a mixture of DNA molecules of different composition of bases, but that the same mixture of DNA molecules may be present in all nuclei of one species. In any event, it is reasonable to assume that the RNA and DNA molecular complement of an individual cell must vary either in relation to the proportion or sequence (or both) of bases.

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After Dr. Levius presented the paper there was a DISCUSSION.

AN INVESTIGATION INTO THE EFFECTIVENESS OF VARIOUS ANTOXIDANTS ON THE PRESERVATION OF FRESH GROUNDNUT OIL (FOOD GRADE) B.P.

By G. A. Birchall and R. I. Felix

From the Distillers Company (Biochemicals) Limited, Speke, Liverpool

Received May 23, 1960

Of several compounds examined the n-propyl, n-octyl and n-dodecyl esters of gallic acid alone or in combination with certain chelating agents are the most effective preservatives of groundnut oil.

Vegetable oils will maintain their freshness only for a limited period and upon storage progressively deteriorate, developing a detectable rancidity.

Rancidity may be induced by a number of factors, but the most frequent is spontaneous oxidation of the double bonds by atmospheric oxygen which leads to the formation of organic peroxides.

Reducing agents are effective against oxidising agents and atmospheric oxygen and antoxidants are effective against atmospheric oxygen only. It has been suggested that antoxidants act by disrupting the chain reactions which lead to the production of peroxides[1], and they are of value only when added to oils which have not already deteriorated. In addition, chelating agents such as citric acid and acetone dicarboxylic acid, will complex with contaminating trace metals such as iron or copper which catalyse oxidation, and consequently enhance the action of some antoxidants. Furthermore, the activity of a combination of certain antoxidants is greater than when each is present separately.

An ideal antoxidant should possess the following characteristics.

No toxicity and no irritant properties even after prolonged usage.
No influence on colour, taste or odour of the substrate.
Ready solubility in the substrate.
Effectiveness in low concentration.
Be chemically inactive with other constituents and neutral in reaction.
Be non-volatile thus ensuring no fall in concentration after prolonged storage.

Experimental

Determination of Peroxides

The method was essentially that described by Heaton and Uri[2] but the period of nitrogen sparging and the reaction time was reduced.

Reagents. Solvent solution. The solution consists of two volumes of glacial acetic acid B.P. and one volume of carbon tetrachloride, and is stored in a closed container and used within three days of preparation. *Saturated solution of potassium iodide.* A saturated solution of potassium iodide in distilled water. 0·002N *Sodium thiosulphate.* Freshly prepared. *Starch indicator.* 1 per cent w/v soluble starch in distilled water.

Method of Peroxide Determination

Six boiling tubes are arranged in series and connected by a reducing valve to a source of oxygen-free nitrogen. Accurately weighed samples

TABLE I

EVALUATION OF ANTOXIDANTS IN SAMPLES OF GROUNDNUT OIL (FOOD GRADE) B.P. STORED AT 37° RECORDING PEROXIDE VALUE AGAINST STORAGE INTERVAL IN DAYS

	Antoxidant	w/v per cent	Sample of oil	Storage interval in days																						
				0	6	11	14	26	27	28	30	41	46	50	55	66	67	73	74	82	90	91	92	105	122	134	144	323
1.	Propyl gallate	0·01	A	4·2	5·5	—	—	—	6·6	—	—	8·8*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2.	Propyl gallate	0·015	A	4·5	5·3	—	—	—	6·2	—	—	7·3	—	—	—	—	—	—	—	9·9	—	—	—	11·3	—	12·1	—	—
3.	Propyl gallate	0·02	A	4·2	5·6	—	—	—	5·9	—	—	7·8	—	—	—	—	—	—	—	8·6	—	—	—	10·7	—	11·2	—	—
4.	Propyl gallate } Citric acid }	0·015 0·01	A	4·1	5·9	—	—	—	5·7	—	—	7·1	—	—	—	—	—	—	—	8·6	—	—	—	10·4	—	10·1	—	—
5.	Octyl gallate	0·01	A	4·2	6·7	—	—	—	7·8	—	—	11·5*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6.	Octyl gallate	0·015	A	4·3	5·8	—	—	—	6·7	—	—	8·5	—	—	—	—	—	—	—	10·1	—	—	—	11·1	—	13·8	—	—
7.	Octyl gallate } Citric acid }	0·015 0·01	A	4·5	5·7	—	—	—	5·8	—	—	7·7	—	—	—	—	—	—	—	8·9	—	—	—	11·7	—	12·7	—	—
8.	Control	—	A	4·8	7·9	—	—	—	12·4	—	—	19·0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9.	Dodecyl gallate	0·01	B	2·4	—	—	—	—	—	—	6·5	—	—	—	7·4	—	—	—	—	—	—	—	6·7	—	10·0	—	—	30·8
10.	Dodecyl gallate	0·015	B	4·7	—	—	—	—	—	—	6·0	—	—	—	6·1	—	—	—	—	—	—	—	9·8	—	10·6	—	—	17·3
11.	Dodecyl gallate } Citric acid }	0·015 0·01	B	2·9	—	—	—	—	—	—	7·7	—	—	—	7·0	—	—	—	—	—	—	—	7·5	—	9·4	—	—	17·5
12.	Propyl gallate } Citric acid }	0·02 0·01	B	2·8	—	—	—	—	—	—	8·5	—	—	—	5·4	—	—	—	—	—	—	—	7·1	—	7·2	—	—	13·0
13.	Butylated hydroxy toluene } Butylated hydroxy anisole }	0·01 0·01	B	4·9	—	—	—	—	—	—	7·2	—	—	—	7·6	—	—	—	—	—	—	—	12·5	—	21·8	—	—	—
14.	Butylated hydroxy toluene } Butylated hydroxy anisole }	0·02 0·02	B	6·0	—	—	—	—	—	—	8·1	—	—	—	8·6	—	—	—	—	—	—	—	29·3	—	—	—	—	—
15.	Butylated hydroxy anisole } Propyl gallate } Citric acid }	0·02 0·006 0·004	B	3·4	—	—	—	—	—	—	6·0	—	—	—	6·9	—	—	—	—	—	—	—	16·4	—	21·2	—	—	—
16.	Propyl gallate	0·02	B	3·0	—	—	—	—	—	—	5·2	—	—	—	—	—	—	—	—	—	—	—	5·4	—	6·5	—	—	14·2
17.	Control	—	B	5·9	—	—	—	—	—	—	15·2	—	—	—	16·6	—	—	—	—	—	—	—	>30	—	—	—	—	—
18.	Butylated hydroxy anisole	0·01	C	6·2	—	—	7·2	12·4*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
19.	Butylated hydroxy anisole	0·015	C	7·3	—	—	10·4	14·8*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20.	Butylated hydroxy anisole	0·02	C	7·1	—	—	11·6	13·9*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
21.	Butylated hydroxy toluene	0·01	C	7·6	—	—	8·8	10·1*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22.	Butylated hydroxy toluene	0·015	C	7·4	—	—	7·8	8·7	—	—	—	—	9·2	—	—	—	26·1	—	—	—	—	—	—	—	—	—	—	—
23.	Butylated hydroxy toluene	0·02	C	7·9	—	—	9·1	9·4	—	—	—	—	7·9	—	—	—	15·3	—	—	—	16·2	—	—	—	—	—	—	—
24.	Control	—	C	6·7	—	—	12·1	14·2	—	—	—	—	20·3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
25.	Acetone dicarboxylic acid	0·02	D	0·9	—	4·2	—	—	—	11·3	—	—	—	11·8	—	16·6	—	18·0	21·7	—	—	—	—	—	—	—	—	—
26.	Propyl gallate	0·02	D	0·5	—	2·4	—	—	—	1·8	—	—	—	1·4	—	2·1	—	—	1·6	—	—	1·4	—	—	—	—	2·7	—
27.	Propyl gallate } Acetone dicarboxylic acid }	0·02 0·02	D	1·7	—	2·0	—	—	—	3·8	—	—	—	3·0	—	—	—	1·1	3·4	—	—	2·8	—	—	—	—	3·5	—
28.	Dodecyl gallate	0·02	D	0·8	—	1·8	—	—	—	2·2	—	—	—	1·9	—	—	—	—	3·4	—	—	1·8	—	—	—	—	2·8	—
29.	Control	—	D	1·2	—	1·8	—	—	—	3·1	—	—	—	6·4	—	4·6	—	10·5	12·5	—	—	16·6	—	—	—	—	31·5	—

* Discontinued, ineffective at 60°.

of the oil (about 1 g.) are introduced into tubes 2–5. Tube 1 contains only solvent solution, to saturate the nitrogen stream. 20·0 ml. of the solvent solution is added and nitrogen bubbled through at a constant rate for 30 minutes. 0·5 ml. of potassium iodide solution is introduced into tubes 2–6. The nitrogen is passed for a further five minutes to ensure complete mixing of the solutions. The tubes are then sealed and stored in the dark for 30 minutes. The contents of tubes 2–6 are transferred into conical flasks containing 30 ml. of distilled water and each titrated with 0·002N sodium thiosulphate using starch as indicator. A "blank" determination is carried out on tube 6. The peroxide value is determined by the following equation, titre of sample–titre of blank/wt. of sample in g.

Experimental Method

The additives were dissolved in groundnut oil, 50 ml. of which was put into 2 oz. open glass jars stored at 37° and 60°. The peroxide values were determined at intervals.

Initial determinations were made on all samples and a sample of untreated oil was used as the control. Accelerated tests at 37° and 60° were also made.

Four samples of different batches of oil, obtained from the same supplier were examined.

Antoxidants Examined

The antoxidants examined are those permitted for use in foodstuffs.[3] They were n-propyl gallate, n-octyl gallate, n-dodecyl gallate, butylated hydroxy anisole, butylated hydroxy toluene, combinations of the above, and combinations of the above with citric acid and acetone dicarboxylic acid.

Results

The results have been tabulated under their storage conditions. Table I records the peroxide values obtained at 37° and Table II at 60°.

The quantitative work was stopped when undesirable rancidity was evident. This corresponded to a peroxide value of about 20.

Discussion

Of the antoxidants examined, the esters of gallic acid, alone or in combination with citric acid or acetone dicarboxylic acid, are the most effective in delaying the onset of oxidative rancidity. Propyl gallate 0·02 per cent w/v alone is more efficient than dodecyl gallate 0·02 per cent w/v alone. However, the gallate moiety is the active group and the use of an identical concentration of the higher molecular weight ester on a weight basis, results in a lower content of the gallate radical and consequently, antoxidant activity.

Acetone dicarboxylic acid alone appears to accelerate the rate of oxidation and no marked antoxidant activity is evident even in combination with propyl gallate.

TABLE II

EVALUATION OF ANTOXIDANTS IN SAMPLES OF GROUNDNUT OIL (FOOD GRADE) B.P. STORED AT 60° RECORDING PEROXIDE VALUE AGAINST STORAGE INTERVAL IN DAYS

	Antoxidants	w/v per cent	Sample of oil	Storage interval in days																				
				0	2	3	5	6	7	10	13	17	19	20	21	25	26	31	35	40	47	52	54	55	66
1.	Propyl gallate	0·01	A	4·2	5·3	—	—	5·3	6·6	6·9	—	—	10·5	—	—	—	13·5	—	21·8	—	—	—	—	—	—
2.	Propyl gallate	0·015	A	4·5	5·9	—	—	—	6·7	6·8	—	—	7·8	—	—	—	11·7	—	13·9	16·2	17·1	—	—	33·8	—
3.	Propyl gallate	0·02	A	4·2	5·3	—	—	—	6·6	6·7	—	—	8·1	—	—	—	18·6	—	13·3	17·3	14·7	—	—	20·6	—
4.	Citric acid + Propyl gallate	0·01 + 0·015	A	4·1	5·5	—	—	—	6·4	6·4	—	—	8·7	—	—	—	10·4	—	13·0	14·4	13·6	—	—	16·1	—
5.	Octyl gallate	0·01	A	4·2	5·8	—	—	—	7·1	7·6	—	—	9·5	—	—	—	12·8	—	17·8	27·7	—	—	—	—	—
6.	Octyl gallate	0·015	A	4·3	4·9	—	—	—	6·1	7·6	—	—	9·4	—	—	—	12·4	—	17·5	19·8	—	—	—	—	—
7.	Octyl gallate + Citric acid	0·015 + 0·01	A	4·5	4·9	—	—	—	6·1	4·2	—	—	9·8	—	—	—	11·4	—	14·7	16·4	19·1	—	—	—	—
8.	Control	—	A	4·8	7·7	—	—	—	14·0	16·5	21·3	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9.	Butylated hydroxy anisole	0·01	C	6·2	—	—	—	22·0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10.	Butylated hydroxy anisole	0·015	C	7·3	—	—	—	20·7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11.	Butylated hydroxy anisole	0·02	C	7·1	—	—	—	22·0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12.	Butylated hydroxy toluene	0·01	C	7·6	—	—	—	—	—	—	13·2	—	—	18·3	—	32·1	—	—	—	—	—	—	—	—	—
13.	Butylated hydroxy toluene	0·015	C	7·4	—	—	—	—	—	—	12·1	—	—	17·1	—	23·4	—	—	—	—	—	—	—	—	—
14.	Butylated hydroxy toluene	0·02	C	7·9	—	—	—	—	—	—	11·2	—	—	17·1	—	20·1	—	—	—	—	—	—	—	—	—
15.	Control	—	C	6·7	—	—	—	17·7	—	—	26·2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16.	Acetone dicarboxylic acid	0·02	D	0·9	—	4·3	15·3	—	14·2	36·8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
17.	Propyl gallate	0·02	D	0·5	—	2·3	2·4	—	2·6	2·5	—	4·7	—	—	4·8	—	—	9·2	—	11·0	—	16·0	16·4	—	53·0
18.	Propyl gallate + Acetone dicarboxylic acid	0·02 + 0·02	D	1·7	—	2·3	3·0	—	3·2	4·8	—	7·0	—	—	13·2	—	—	9·7	—	13·3	—	—	—	—	—
19.	Dodecyl gallate	0·02	D	0·8	—	2·1	3·3	—	3·2	4·4	—	5·2	—	—	3·8	—	—	11·5	—	14·6	—	>100	—	—	—
20.	Control	—	D	1·2	—	4·8	5·0	—	10·8	14·1	—	>100	—	—	—	—	—	—	—	—	—	—	—	—	—

Butylated hydroxy anisole and butylated hydroxy toluene, at the concentrations used, either alone or in combination are much less effective than the gallates, butylated hydroxy toluene having a greater activity than butylated hydroxy anisole which has little effect.

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After Mr. Felix presented the paper there was a DISCUSSION.

AN EXAMINATION OF THE DECOMPOSITION OF DEXTROSE SOLUTION DURING STERILISATION

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A method is described for assessing the degree of decomposition of dextrose in solutions by measuring absorptiometrically the blue colour produced by a reaction with Folin-Ciocalteu reagent. The amount of decomposition depends on the duration and temperature of the sterilisation process, the concentration of dextrose and the presence of added substances. It is minimal at about pH 3. A product other than 5-hydroxymethylfurfural is formed. Two breakdown products of dextrose, gluconic acid and 5-hydroxymethyfurfural, also progressively alter in composition when their solutions are autoclaved for increasing periods either separately or as a mixture.

Solutions of dextrose are known to undergo change when heated: this is often associated with the development of a straw colour. Webb and others[1] found that 5-hydroxymethylfurfural arose from a general acid-base hydrolysis of dextrose in solution and that the rate of reaction was inversely proportional to the initial concentration of the dextrose. Singh and others[2] state that the degree of colouration paralleled the extent of formation of 5-hydroxymethylfurfural, and Scallat and Gardner[3] have suggested that the colour is at least partly due to the polymerisation of this substance. Hirayama and Kubuta[4] have reported negligible decomposition at 100° which rapidly increases when solutions are sterilised under pressure. Hudson and Tarlowski[5] noted the lowering of the pH of dextrose solutions as a result of sterilisation, and Singh and others[2] found decomposition was minimum at pH 3. Griffen and Marie[6] showed that in the presence of sodium lactate the decomposition was increased, but was at a minimum at pH 5. Hornauer[7] has shown that the pH falls with increasing rise of temperature and strength of solution.

Colour Reaction Developed by Sterilised Dextrose Solutions with Folin-Ciocalteu Reagent

Sterilised solutions of dextrose react with Folin-Ciocalteu reagent to give a stable blue colour, and this has been adapted for measuring the degree of decomposition of these solutions. To 4 ml. of a solution of dextrose in a test tube was added 0·6 ml. of Folin-Ciocalteu reagent and 1·0 ml. of 25 per cent solution of sodium carbonate and the whole mixed. The tube was placed in a water bath at 37° and heated for 15 minutes. A blank solution of 4 ml. of water was similarly treated. Readings were then made in a Spekker absorptiometer using filter No. 608.

Action of Added Substances upon Folin-Ciocalteu Reaction

To determine whether substances used with dextrose given by intravenous infusion affected the colour, measurements were made with a

solution of phenol 0·001 per cent w/v containing the added substances in the concentration used in solutions of dextrose. Sodium acid citrate, potassium phosphate, sodium lactate and hydrochloric acid did not alter the colour reaction. Sodium metabisulphite effected a much deeper colour and therefore solutions containing it cannot be determined by this method.

Measurements of solutions of 5-hydroxymethylfurfural of known strength and of unheated solutions of dextrose 5 per cent containing the same concentrations of 5-hydroxymethylfurfural were also made. The absorption readings for the combined solutions were found to be additive.

Nature of the Reaction

As the decomposition of dextrose solutions has been related to the formation of 5-hydroxymethylfurfural, solutions of the latter were subjected to the method described and were found to give a blue colour

TABLE I

COMPARISON OF TWO METHODS OF ASSESSING THE DECOMPOSITION OF DEXTROSE SOLUTIONS BY REFERENCE TO THE CONTENT OF 5-HYDROXYMETHYLFURFURAL

Sample No.	Strength of solution	5-HMF content per cent w/v.	
		(*a*) Direct absorption method	(*b*) Folin-Ciocalteu reaction method
322	5 per cent w/v	0·00014	0·465
329	10 per cent w/v	0·000365	0·72
301	20 per cent w/v	0·00084	1·095

which was directly proportional to the strength of the solution. The colour changed slightly on long standing, whereas that produced by dextrose solutions remained unchanged. By relating the colours produced in dextrose and 5-hydroxymethylfurfural solutions the degree of decomposition after sterilising appeared far in excess of that reported by others. The method was therefore compared with direct absorption in a Beckmann D.K.2 spectrophotometer. As seen in Table I, the results obtained by direct absorption indicated the formation of much smaller amounts of 5-hydroxymethylfurfural than those estimated by the method using Folin-Ciocalteu reagent. This suggested that at least one other substance which produces a much more intense blue colour with this reagent is present, and it appears to be a further product of decomposition of dextrose.

Solutions of gluconic acid and 5-hydroxymethylfurfural, both decomposition products of dextrose, produce a blue or more intense blue colouration with Folin-Ciocalteu reagent after autoclaving. Separately and in mixtures both substances were found to change progressively after autoclaving for 30 minutes successively 8 times, producing another substance which may be similar to that formed during the sterilisation of dextrose solutions. With gluconic acid, the blue colour develops after heating a solution under pressure. Further autoclaving, as with 5-hydroxymethylfurfural, produces a more intense blue colour.

Examination of Sterilised Dextrose Solutions

Using the method detailed, solutions of dextrose were examined and the readings used to compare the degree of decomposition. As many of the solutions were in containers fitted with rubber closures, water and normal saline solutions prepared under similar conditions were also examined. The results in Table II show that the blue colour arises almost entirely from the decomposed dextrose and that the occasional detection of extracted substance from rubber-capped containers arises probably

TABLE II

EXAMINATION OF THE DEGREE OF DECOMPOSITION OF STERILISED DEXTROSE SOLUTIONS

Sample No.	Type of solution	Container	Reading or calculated reading	
31	Normal saline	R.C.	0·000	
32	Normal saline	R.C.	0·060	Manufacturer A
68	Normal saline	R.C.	0·003	Manufacturer B
53	Isotonic Sod. Lactate.	R.C.	0·008	Manufacturer C
55	Isotonic Sod. Lactate.	R.C.	0·013	
13	4·3 per cent Dextrose with 0·18 per cent sod. chloride	R.C.	0·466	
33	4·3 per cent Dextrose with 0·18 per cent sod. chloride	R.C.	0·537	Manufacturer A
70	4·3 per cent Dextrose with 0·18 per cent sod. chloride	R.C.	0·271	Manufacturer B
17	5 per cent Dextrose	R.C.	0·377	
214	5 per cent Dextrose	R.C.	0·670	Manufacturer A
218	5 per cent Dextrose	R.C.	0·368	Manufacturer B
51	5 per cent Dextrose	R.C.	0·215	Manufacturer C
472	5 per cent Dextrose	R.C.	0·362	Manufacturer C
26	50 per cent Dextrose	Ampoules	1·335	Manufacturer D
45	50 per cent Dextrose	Ampoules	1·04	Manufacturer D
46	50 per cent Dextrose	Ampoules	2·35	Manufacturer E

R.C.—fitted with rubber closure.

TABLE III

EXAMINATION OF UNHEATED SOLUTIONS OF DEXTROSE OF COMMERCE

Sample No.	Type	Strength per cent	Reading	pH of solution
25	Dextrose B.P.	5 w/v	0·040	
127	Dextrose B.P.	5 w/v	0·035	
431	Dextrose B.P.	10 w/v	0·223	5·18
432	Dextrose B.P.	10 w/v	0·176	
433	Dextrose B.P.	10 w/v	0·230	5·26
434	Dextrose B.P.	10 w/v	0·194	
435	Dextrose B.P.	10 w/v	0·220	5·20
436	Dextrose B.P.	10 w/v	0·197	
451	Dextrose B.P.	10 w/v	0·090	
437	Dextrose analytical grade	10 w/v	0·142	5·13
438	Dextrose analytical grade	10 w/v	0·118	
439	Dextrose monohydrate	10 w/v	0·115	5·20
440	Dextrose monohydrate	10 w/v	0·107	

from the inadequate treatment of closures before use, and contributes only a small amount of blue colour to dextrose solutions.

To determine what factors influenced the formation of the decomposition product giving a blue colour with Folin-Ciocalteu reagent a systematic examination was undertaken.

Dextrose Powder

Solutions of Dextrose B.P. and the monohydrate prepared without heat were examined for the substance yielding a blue colour to Folin-Ciocalteu

reagent. Table III shows that variable amounts are present and could influence the results obtained with the sterilised solutions.

Effect of Concentration of Dextrose

An aqueous 20 per cent solution of dextrose was prepared without heat. This together with dilutions containing 5 per cent and 10 per cent dextrose were filled into 500 ml. containers which were either sterilised for 30 minutes at 10 lb. or heated for 1 hour at 98–100°. The results in Table IV show that the amount of decomposition is much increased by raising the

TABLE IV

EFFECT OF CONCENTRATION AND TEMPERATURE ON THE DECOMPOSITION OF DEXTROSE SOLUTIONS

Sample No.	Strength of solution per cent w/v	(*a*) Unheated solution	(*b*) Autoclaved for 30 min. at 10 lb. pressure		(*c*) Heated for 1 hour at 98–100°	
			Reading	(*b*)–(*a*)	Reading	(*c*)–(*a*)
323	5	0·110	0·428	0·318	—	—
330	10	0·220	0·651	0·431	—	—
304	20	0·448	0·962	0·514	—	—
324	5	0·110	—	—	0·157	0·047
347	10	0·220	—	—	0·380	0·160
305	20	0·448	—	—	0·679	0·231

TABLE V

RELATIONSHIP OF DECOMPOSITION TO LENGTH OF TIME OF THE HEATING PROCESS IN SOLUTIONS OF DEXTROSE 5 PER CENT

Sample No.	Heating	Reading
35	30 min. at 10 lb. pressure	0·302
37	45 min. at 10 lb. pressure	0·347
36	30 min. at 10 lb. pressure and overnight storage in autoclave	0·424
38	45 min. at 10 lb. pressure and overnight storage in autoclave	0·405
43	45 min. at 10 lb. pressure and overnight storage in full autoclave	0·583
131, 132, 147, 148, 219,	30 min. at 10 lb. in ampoule	0·392 (average)
133, 134, 149, 150, 220,	2 × 30 min. at 10 lb. in ampoule	0·554 (average)
135, 136, 151, 152, 221,	4 × 30 min. at 10 lb. in ampoule	0·833 (average)
137, 138, 153, 154.	8 × 30 min. at 10 lb. in ampoule	1·392 (average)

temperature of the solution, and that the effect of increasing the concentration of the dextrose is to raise the total amount of decomposition product but to reduce the rate of formation. This is in agreement with Webb and others[1] and Hirayama and Kubuta[4].

Time Factor

During the examination of 5 per cent solutions of dextrose, more decomposition appeared after autoclaving for 45 minutes, than for 30 minutes. Also more decomposition occurred when batches were left to stand overnight in the autoclave instead of removing as soon as practicable. This is seen in Table V.

Further investigation with ampoules of 5 per cent dextrose solution subjected to autoclaving at 10 lb. pressure for 30 minutes for one, two,

four and eight times, showed that the amount of decomposition was directly related to the length of time of autoclaving (Table V).

Presence of other Substances and Effect of pH

Singh and others[2] showed that the decomposition of dextrose solutions as measured by 5-hydroxymethylfurfural formation was least in solutions with a pH of 3 before autoclaving. Webb and others[1] also showed that the rate of decomposition was related to the concentration of buffer at a constant pH.

Hydrochloric acid and a number of substances commonly used in intravenous infusions were added to dextrose solutions. The solution containing 0·01 per cent w/v HCl showed less than one-quarter of the decomposition shown by an unmodified solution of dextrose, the pH remaining constant at 2·62. A solution with 0·0001 N HCl was less stable than the above but more stable than the unmodified solution, the pH moving from 4·16 before to 3·73 after autoclaving. Sodium chloride

TABLE VI

EFFECT OF ADDED SUBSTANCES ON THE STABILITY OF DEXTROSE SOLUTIONS

Sample No.	Strength of dextrose w/v	Strength of added substance w/v per cent	pH		Reading or calculated reading		
			Before autoclaving	After autoclaving	(*a*) Autoclaved solution	(*b*) Unheated solution	(*a*)-(*b*)
323	5	—	5·28	3·97	0·428	0·11	0·318
342	5	0·01 HCl	2·62	2·62	0·183	0·11	0·073
353	5	0·000365 HCl	4·16	3·73	0·330	0·11	0·220
333	5	0·9 NaCl	5·40	3·94	0·448	0·11	0·338
319	5	0·22 Sod. lactate	5·93	5·41	0·964	0·11	0·854
315	5	0·05 K_2HPO_4	7·89	5·09	4·420	0·11	4·310
311	5	4·18 Sod. acid citrate	4·92	4·91	0·586	0·11	0·476
343	2·5	2·09 Sod. acid citrate	5·04	5·08	0·195	0·055	0·140

0·9 per cent has no effect on decomposition or the pH shift. Potassium phosphate at 0·05 per cent w/v caused a marked decomposition associated with the development of a definite brown colour. As the initial pH was 7·89 this was to be expected. A solution containing 0·22 per cent w/v of sodium lactate also caused increased decomposition. Sodium acid citrate used with dextrose at the concentration used for collecting blood produces a fairly stable solution. However, by comparing this solution with one of twice its strength it was found that the increase in concentration of the buffer accentuates the decomposition of the dextrose. Also, although the pH remained constant, heating for half an hour at 20 lb. pressure produced a twenty-five-fold increase in the decomposition as measured by the method detailed.

Unbuffered dextrose solutions normally become more acid during sterilisation to a point about pH 4. The addition of buffers in low concentration capable of maintaining the pH at 4 provides some stability, but higher concentrations increase the rate of decomposition. Added substances giving a pH which alters during sterilisation also appear to increase the formation of breakdown products of dextrose (Table VI).

Discussion

It may be deduced that the decomposition of dextrose is directly related to the length and temperature of the sterilising process. Also, samples of dextrose powder vary in the amount of the product or products of degradation present, although in all experiments this was small.

The addition of hydrochloric acid to pH 3, as shown by other workers, also appears to be the most satisfactory means of reducing decomposition chemically. Because of the increase in decomposition, sterilisation of dextrose solutions in the presence of other substances is inadvisable. To attain minimum decomposition care must be taken to ensure that the heating process is not prolonged beyond the time necessary for sterilisation, and that no overheating takes place. At the same time it is an advantage to cool the solution as quickly as possible after sterilising.

With Folin-Ciocalteu reagent, results showed the presence of a substance other than 5-hydroxymethylfurfural, and it appears that another detectable decomposition product is present, probably in amounts comparable with the amount of 5-hydroxymethylfurfural determined by direct spectrophotometric absorption. Certainly the results obtained in this work parallel observations made by others who related decomposition to the formation of 5-hydroxymethylfurfural. The development of more intense blue colour reactions in solutions of both gluconic acid and 5-hydroxymethylfurfural, subjected to progressively longer periods of sterilisation, show also that these products of dextrose decomposition produce other substances during such treatment. However, as relatively strong solutions of these substances were used, this may only be taken as an indication, and not the explanation of what is happening in the alteration of the dextrose molecule under the conditions of sterilisation of solutions of that substance.

Acknowledgements. I wish to acknowledge the assistance of Miss S. McDowell in undertaking much of the analytical work, the co-operation of Dr. J. R. Gwilt and Mr. E. R. Brown of Winthrop Laboratories Limited in undertaking determinations with the Beckmann Absorptiometer, and also Dr. B. E. Tomlinson and Mr. G. B. Pendleton for the use of the Spekker Absorptiometer.

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After Mr. Wing presented the paper there was a DISCUSSION.

A SHORTER STERILISING CYCLE FOR SOLUTIONS HEATED IN AN AUTOCLAVE

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The rapid cooling of bottles in an autoclave after steam sterilisation has been investigated. A coarse cold water spray caused breakage by thermal shock, whereas a fine spray prevented this. The time of cooling using this method has been compared with the usual cooling cycle and with that achieved when air was circulated in the chamber. Experimental data for a steriliser containing a charge of 200 × 1 litre bottles is given.

Sterilisation of fluids by heating in an autoclave is normally a lengthy process which may be divided into three phases. The first is the time required to heat the chamber and contents to the sterilising temperature; this is dependent upon the water-equivalent of the steriliser and its contents, the quality of the incoming steam and the condition of the autoclave. Secondly, there is the sterilising period, which is set by the temperature selected for the operation[1,2]. Thirdly, there is the cooling time which is dependent upon the heat capacity of the system and the rate at which heat can be dissipated.

When a steriliser, designed so that heat losses are small, is used with dry saturated steam the heating time is a minimum; the sterilising time at a given temperature may not be shortened, so only the cooling period may be reduced.

Reduction of the cooling period offers the advantages of safety, improvement of product and reduction in costs. For example, when the chamber has reached atmospheric pressure the contents of closed bottles, because of their slow rate of cooling, have an internal pressure above atmospheric pressure, and an explosion may occur or the closure may be blown off.

Hitherto the rate of cooling has been dependent upon the heat transfer of the system to the surrounding air by conduction and radiation: by the means now described the containers are cooled directly. The idea of rapidly cooling large volumes of fluids is not new. An autoclave has been described[3] in which streams of warm water are directed onto the hot containers, but elaborate temperature control of the water is necessary to avoid breaking the bottles. We report a method of reducing the cooling period using cold water without recourse to temperature control systems.

Experimental

The steriliser and recording apparatus have previously been described[4] and in addition an unjacketed autoclave measuring 122 × 114 cm. diameter (48 × 45 in.) was used for confirmatory experiments. Modifications were made so that water under pressure was supplied to spray nozzles within the chambers. Compressed air was also supplied to

ballast the chambers at sterilising pressure as soon as the incoming water condensed the steam. A relief valve set to open at 2 p.s.i. above working pressure was fitted.

Two spray nozzles were positioned opposite each other at either end of the chamber and pointing slightly downwards. They were also off-set in the horizontal plane to produce maximum turbulence (Fig. 1). In the larger steriliser 12 nozzles were positioned at the top of the chamber in three rows of four pointing directly downwards. These nozzles were of the whirling-spray mechanical break-up type.

Deionised or distilled water was necessary to obtain deposit-free surfaces. The rate of flow through the nozzles was measured, and at the working pressure of 100 p.s.i. (7·03 kg./sq. cm.) was 0·5 litres each per minute.

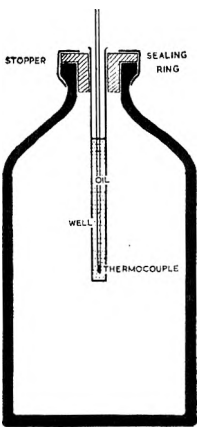

FIG. 1. Arrangement for the insertion of a thermocouple in a transfusion bottle.

To avoid repeated blocking of the nozzles by material flaking from the inner surface of iron or galvanised iron pipes, copper piping was used in the water circuit.

Temperature Measurement

Transfusion bottles. Thermometer wells, filled with high-density oil[5] were produced so that the thermocouple junctions were about the centre of the bottles (Fig. 2).

Ampoules. The neck of a standard 10 ml. ampoule was cut and the cut end flattened to form a lip. A rubber bung, through which the thermocouple wires passed, was inserted into the ampoule neck and wired on. *Vials.* Standard 10 ml. vials were used. Thermocouple wires were passed through a pierced latex plug. *M.R.C. bottles.* The plug was pierced and the thermocouple wires passed through until the junction was below the surface of the liquid. Three such containers were usually used, one at each end of the chamber and the third about the middle. In the larger unjacketed autoclave, in which the charge consisted of three layers of bottles, a bottle was selected near the centre of each layer for temperature measurement.

Measurement of Droplets from Nozzles

A slight variation of an existing method[6] was used. A standard glass microscope slide, 3 in. × 1 in., was evenly coated with magnesium oxide and exposed by a shutter to the spray for a fraction of a second. The droplets caused areas of the oxide film to be disturbed; measurement of the mean diameter of these areas provided a measure of droplet size.

Operating Conditions

Using dry saturated steam at 10 p.s.i. (0·703 kg./sq. cm.) normal practice was followed to the end of the holding period. At this point water was supplied to the spray nozzles at their working pressure, and pressure maintained in the chamber by compressed air. When the liquid in the bottles

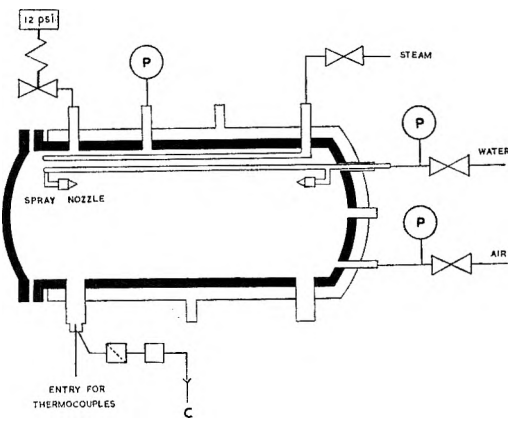

FIG. 2. General arrangement of the steriliser with water spray. Graphical symbols B.S. 1553.

had cooled to its boiling point the supply of air was discontinued and further cooling to 93° (200° F.) achieved by water alone. To prevent waterlogging of the chamber, a steam trap capable of passing the full water flow was fitted.

RESULTS

Experiments with different nozzles indicated that the maximum droplet size became more and more critical as the temperature of the cooling water was reduced: there was no advantage in using a smaller droplet size than that which just fails to crack bottles, as with smaller nozzles the necessary flow of water to the chamber is impeded: at a water temperature of 18° (65° F.) a mean droplet size of 80 μ produced the maximum rate of cooling.

Certain positions of the nozzles caused breakages. Coalescence of small droplets on the upper surface of the chamber caused large drops to fall on to the bottles and produce a thermal shock. With suitably positioned nozzles the major factors associated with the cooling of the

charge were shown to be the water equivalent of the bottles and their contents, and the rate of heat transfer through their walls. Measurements were made with glass and aluminium containers (Fig. 3).

Rates of Cooling

Transfusion solutions. Control experiments showed that with a charge of twenty-four 0·5 litre bottles in the smaller autoclave, 3 hours elapsed before the temperature reached 93° (200° F.) (Fig. 4). In the larger steriliser with a 200 × 1 litre charge, 22 hours were needed to reduce the temperature to 93° (200° F.) (Fig. 5). In other experiments with the smaller autoclave, air was pumped through at the end of the holding period. This process may be described as "air-cooling" and by this means the time to 93° (200° F.) was decreased to 2 hours (Fig. 4).

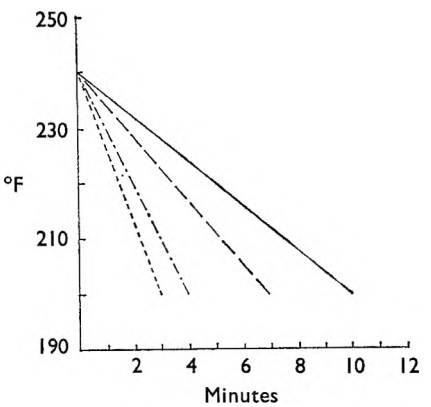

FIG. 3. Water cooling. Comparative rates of cooling of containers.

——— 0·5 l. transfusion, glass
– – – – M.R.C. bottles
–·–·– 0·5 l. transfusion, aluminium
------ Ampoules and vials

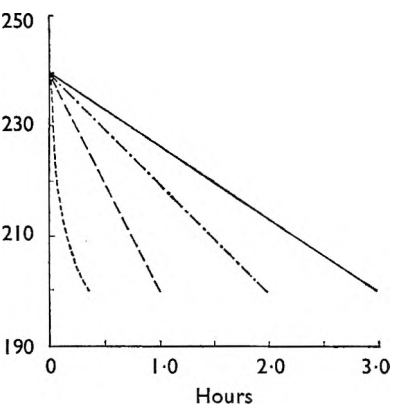

FIG. 4. Natural cooling. Comparison of the rates of cooling of containers under the effect of convection and radiation cooling and without forced circulation.

——— 0·5 l. transfusion free-cooled
–·–·– 0·5 l. transfusion air-cooled
– – – – M.R.C. bottles free-cooling
------ Ampoules and vials free-cooling

When water cooling was employed with the smaller steriliser, the cooling time was decreased to 10 minutes (Fig. 3) and using the larger steriliser a mere 17 minutes reduced the temperature to 93° (200° F.) (Fig. 5). The glass bottles used have survived more than 50 successive treatments. Aluminium containers of similar size and shape to the bottles needed only 4 minutes to cool to 93° (200° F.) compared with 10 minutes for glass (Fig. 3). This confirms that the thermal conductivity of the walls of the containers is a factor limiting the rate of cooling.

Ampoules. Under unassisted cooling conditions, a charge of 10 ml. ampoules required 20 minutes to reach 93° (200° F.) (Fig. 4). Water cooling reduced this time to 3 minutes (Fig. 3). *Vials.* With 10 ml. vials, the results were similar to those obtained with ampoules (Figs. 3 and 4). *M.R.C. bottles.* A charge of 24 × 0·5 litre bottles each containing

120 ml. of solution was used in the smaller autoclave and an unassisted cooling time of 60 minutes recorded (Fig. 4). Water cooling reduced this time to 7 minutes (Fig. 3).

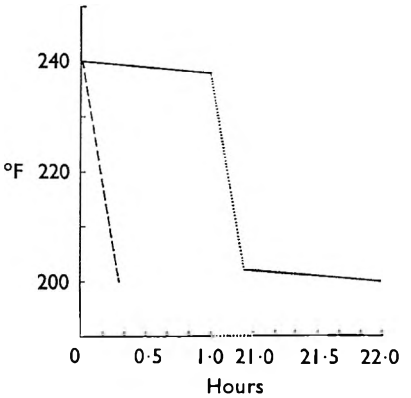

FIG. 5. Cooling curves of 200 one litre bottles of fluid with and without water spray.
——— Free cooling – – – – Water cooling

Effect of Rapid Cooling on Dextrose Injection

One litre bottles containing 5 and 20 per cent Dextrose Injection were processed under normal conditions and also with water cooling. The colour of the solutions was matched against suitable strengths of potassium

TABLE I

EFFECT OF PROCESSES ON DEXTROSE INJECTION

Solution	Treatment	Appearance	Equivalent potassium dichromate
Dextrose injection, 20 per cent ..	Normal	Straw yellow	7 p.p.m.
	Water cooled	Pale yellow	4 p.p.m.
Dextrose injection, 5 per cent ..	Normal	Pale yellow	4 p.p.m.
	Water cooled	Extremely pale yellow	1·5 p.p.m.

dichromate solution as well as being examined visually. Table I shows the results obtained and it is evident that much less discoloration occurs when water cooling is employed.

DISCUSSION

All results show a reduction in the cooling time when the water-cooling method is used. When containers of half or 1 litre transfusion solutions are considered this reduction is large.

It was 22 hours before the temperature of 200 × 1 litre bottles within an unlagged steriliser fell from 115° (240° F.) to 93° (200° F.). The danger of bursting persists until this lower temperature is reached, and presents a hazard if such a batch is removed from the steriliser as soon as the pressure within the chamber has fallen to atmospheric level.

The rate of cooling is limited by the presence of a maximal temperature gradient through the glass walls of the container. If this is exceeded the

glass fractures. The heat capacity of a large cold droplet landing on a hot glass surface is sufficient to bring about the condition of thermal shock. To abstract heat without breaking the bottles it has been found necessary to drench them in a mist of water particles small enough to avoid severe localised cooling and the thermal shock which causes the breakage. Similar experiences have been recorded using standard M.R.C. bottles containing the volume of solution used for blood collection. Vials and ampoules behave in a similar manner but their cooling rate is much higher, because of their greater specific surface and their thinner walls.

Since on a production scale leakage may take place within the steriliser, the closed circuit for the cooling water described previously[3] was discarded. The carbonisation of dextrose solution and the build up of chloride and other salts in the circulating water, which would occur, produces tenacious deposits on the bottle and a risk of corrosion of the steriliser. The optimum droplet size seems to be about 50–100 μ.

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After Mr. Wilkinson presented the paper there was a DISCUSSION. The following points were made.

The positive pressure produced in a large steriliser by steam fell only slowly when the water was introduced due to the "flash" steam production. Only a small amount of air was required to ballast the chamber when the incoming water condensed the steam. The air was fed into the base of the steriliser as a matter of convenience, and had proved the best arrangement, since the air had a greater density than the vapours present and did not interfere with the spray pattern. No shearing of the base of the bottles was caused. The heating and cooling did not exert much strain on the autoclave, and was well within the tolerance of the metal. For insurance purposes a relief valve had to be fitted so that no vacuum was produced. There was no leakage of liquid, either in or out of full or partially filled bottles. In some instances the spray water caused discolouration of aluminium caps if they were in contact with iron.

SOME FACTORS INVOLVED IN MULTIPLE SPOT FORMATION IN THE PAPER CHROMATOGRAPHY OF SYMPATHOMIMETIC AMINES IN THE PRESENCE OF ACIDS*

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The paper chromatographic behaviour of a variety of amines both alone and in the presence of acids in acidic, neutral and basic running solvents is reported. Using a n-butanol: acetic acid: water solvent, multiple spot formation by several pure sympathomimetic amines was observed in the presence of the stronger organic acids. Many weaker acids affected the amines in a neutral solvent. The results emphasise the need for correct controls and the cautious interpretation of chromatograms of biological extracts since the number of spots produced is not necessarily indicative of the number of bases present.

Multiple spot formation in the paper chromatography of pure substances has often been reported[2]. Although satisfactory explanations of the phenomenon in terms of isomerisation, complexing and chemical change for some observations have been given[2,3], the mutual effects of charged ions leading to spot deformation and multiplication in the chromatography of bases have not received detailed attention. However, the effects of acids other than those present in the solvent on the formation of base spots have been noted. For example, Waldron-Edward[4] reported that D-glucosamine and some basic amino-acids and diamines gave two connected base spots in the presence of an equivalent of sulphate, while more than one equivalent resulted in the formation of only the slower base spot. West[5] showed that some pure tissue amines gave two spots when chromatographed in the presence of certain acids. Gore and Adshead[6] found that the R_F values of certain alkaloids were lower when the salts rather than the free bases were used, whereas Resplandy[7] and Büchi and Schumacher[8] reported that the alkaloids and their salts gave identical R_F values in their systems. The use of the same acid in salt and solvent system by Munier[9] resulted in compact spots in the chromatography of diverse bases in accordance with predictions.

In the paper chromatography of biological fluids or extracts, the presence of acids in these materials may alter the R_F values of the bases being examined and give rise to more base spots than there are bases. Quantitative determination of bases by paper chromatography of biological materials may also be seriously jeopardised by the presence of traces of acids. The relative importance of various factors which might be important in the polar interactions involved during the formation of base spots was therefore investigated. In the present paper, sympathomimetic amines only are considered but many of the observations are equally applicable to other amines; qualitative but not quantitative implications in the examination of biological fluids will be presented.

* See reference 1 for a preliminary report of this and related work.

Experimental Methods

Materials. (−)-Noradrenaline acid tartrate was recrystallised from water, m.p. 103° (102–104°[10]). (−)-Adrenaline acid tartrate was recrystallised from water, m.p. 147–148° decomp. (147–154° decomp.[11]). (±)-Isoprenaline sulphate was recrystallised from acetone-methanol, m.p. 178° (180°[11]). Noradrenaline and adrenaline were liberated from solutions of the salts by the addition of dilute ammonium hydroxide solution containing a trace of sodium metabisulphite; the precipitate was filtered off, washed with water, methanol and ether and dried. Noradrenaline gave m.p. 214–216° (216–218°[10]); adrenaline gave m.p. 210° decomp. (211–212°[11]). Isoprenaline was obtained similarly and recrystallised from ethanol, m.p. 155° (155·5°[11]). Tyramine was recrystallised from ethanol, m.p. 163° (164·5°[11]). (−)-Ephedrine hemihydrate was recrystallised from water, m.p. 40° (40°[11]). β-Phenylethylamine and (+)-amphetamine were redistilled; b.p. 196–198° (197–198°[11]) and 200–204° (205°[11]) respectively.

Solvents. (1) n-Butanol : acetic acid : water (4 : 1 : 5, by volume). The liquids were shaken together and set aside overnight. The upper layer was separated off and used as the running solvent.

(2) n-Butanol : ammonia : water (20 : 1 : 19, by volume) was prepared similarly and the upper alcoholic layer utilised.

(3) Water-saturated n-butanol.

(4) Liquefied Phenol, B.P.

Spray Reagents. All solutions were aqueous unless otherwise stated. For *adrenaline*, *noradrenaline* and *isoprenaline*: a solution containing 0·6 per cent w/v potassium ferricyanide and 0·5 per cent w/v sodium hydroxide. For *tyramine*, *β-phenylethylamine*, *ephedrine*: ninhydrin (0·2 per cent w/v) in n-butanol; the papers were dried and heated at 80–100° for 2–3 minutes. *p*-Nitroaniline diazo reagent[12] was used to detect *amphetamine*.

The acid radicals were detected on duplicate chromatograms. *Sulphate*: barium chloride (0·1 per cent w/v) followed by sodium rhodizonate (0·2 per cent w/v). *Chloride*: silver nitrate (0·5 per cent w/v) followed by irradiation by ultra-violet light. In the presence of phenolic amines, the silver nitrate sprayed paper was washed twice with dilute nitric acid (5 per cent w/v HNO_3), and exposed to hydrogen sulphide gas. *Polybasic organic acids*: Dragendorff's reagent. *Salicylic acid*: ferric chloride solution (1·0 per cent w/v) or alternatively by observing the fluorescence under ultra-violet light. *Other acids*: Several methods were used but the following proved the most satisfactory: an aqueous solution of bromophenol blue (0·04 per cent w/v) containing 5 per cent w/v ethanol applied after drying the papers at 50–60° for several hours to drive off the acetic acid. Potassium iodide (1 per cent w/v) followed by a potassium iodate (1 per cent w/v) starch solution.

General method. The bases (0·2 μ M) were applied to Whatman No. 1 paper* for chromatography from aqueous or alcoholic solution as

* Whatman No. 4, 20, 54 and 3MM, and Whatman No. 1 paper previously washed with the running solvent and dried were also used with similar results.

either the free bases, the salts or as the salts in solutions containing an excess of the acid (usually 0·5N). Chromatography was carried out by the ascending technique for 18 hours at 19 ± 2°; the solvent front advanced about 25 cm. during this time. The R_F values reported in this paper were calculated from the positions of the advanced edges of the spots and are expressed as percentages. The deviation from the mean R_F value is given for those values calculated from six or more observations.

Results

Results Using the n-Butanol: Acetic Acid: Water Solvent

Amines having low R_F *values* (0–50), *for example, adrenaline* (R_F 42) *and noradrenaline* (R_F 35). The results are summarised in Figure 1.

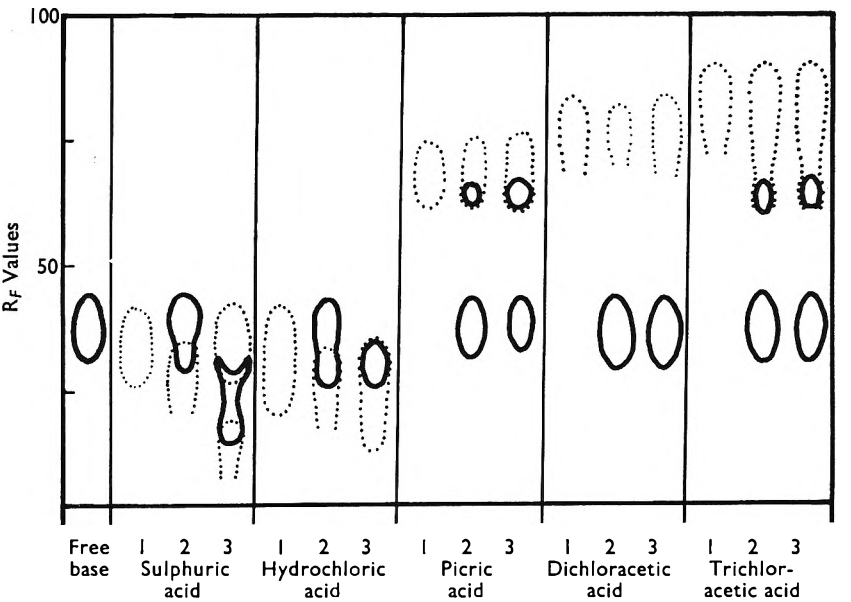

FIG. 1. Chromatograms of adrenaline developed in the butanol : acetic acid : water solvent system when applied to the paper as a suspension (2μl.) of the free base and in solutions (2μl.) containing various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

Both adrenaline (R_F 42 ± 3) and noradrenaline (R_F 35 ± 2) formed elongated amine spots when the hydrochlorides or sulphates were chromatographed. The positions of the leading edges of the amine spots were not significantly different from those obtained when the corresponding free bases were used (see Fig. 1). "Spanner-shaped" amine spots in which part of the acid was present in the cup were produced when either adrenaline or noradrenaline sulphates were applied to the paper with an excess of the acid. The remainder of the acid appeared as an elongated spot below (behind) the amine. This effect was not observed with these

bases in hydrochloric acid when the acid moved in association with the amine as an oval spot having a lower R_F value than that of the free base.

Picrates of adrenaline and noradrenaline yielded two amine spots (R_F 40 and 68 for adrenaline picrate), one of which corresponded with the free base while the other was associated with the picric acid. The R_F value of picric acid is 74 ± 2. Equivalent amounts of weaker organic acids having R_F values similar to that of picric acid, such as maleic acid (R_F 71) and dichloracetic acid (R_F 83) did not affect the movement of adrenaline or noradrenaline although an excess of either acid caused some

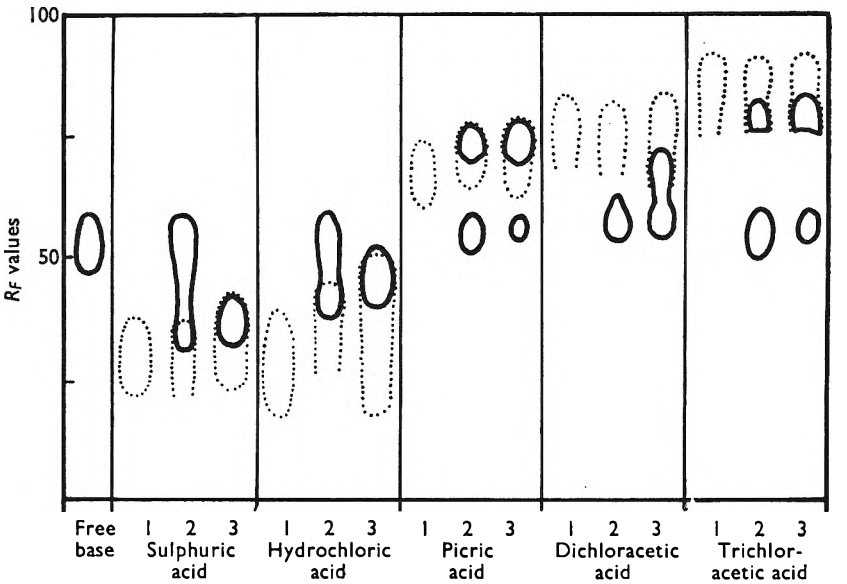

FIG. 2. Chromatograms of isoprenaline developed in the butanol : acetic acid : water solvent system when applied to the paper as a solution (2 μl.) of the free base alone and in the presence of various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

upward streaking with adrenaline. In contrast, stronger organic acids having high R_F values, such as trichloracetic acid (R_F 90 ± 3) and trifluoracetic acid (R_F 82 ± 2), caused formation of two amine spots with both adrenaline and noradrenaline. The slower-moving amine spot corresponded with that of the free base (in equilibrium with the acetic acid of the solvent) while the faster amine spot (R_F of approximately 62) was associated with a halo-acetic acid spot. Increasing the amount of the halo-acetic acid applied with the base resulted in an increasing proportion of the amine in the faster moving spot; the slower amine spot could not be eliminated using up to a twenty molar excess of the acid.

Amines having intermediate R_F *values* (50–70), *for example, isoprenaline* (R_F 59 ± 2) *and tyramine* (R_F 62 ± 2). The results for isoprenaline in

the presence of various acids are presented in Figure 2. When either isoprenaline hydrochloride or sulphate was chromatographed, the base appeared as an elongated spot, the leading edge of which corresponded with that of the more compact spot produced when the free base was chromatographed. The R_F value of the amine was reduced when isoprenaline sulphate was applied to the paper in the presence of excess sulphuric acid (0·5N) (see Fig. 2).

Isoprenaline picrate gave two amine spots, R_F 60 and 79, the former being associated with the picric acid. In contrast with adrenaline and

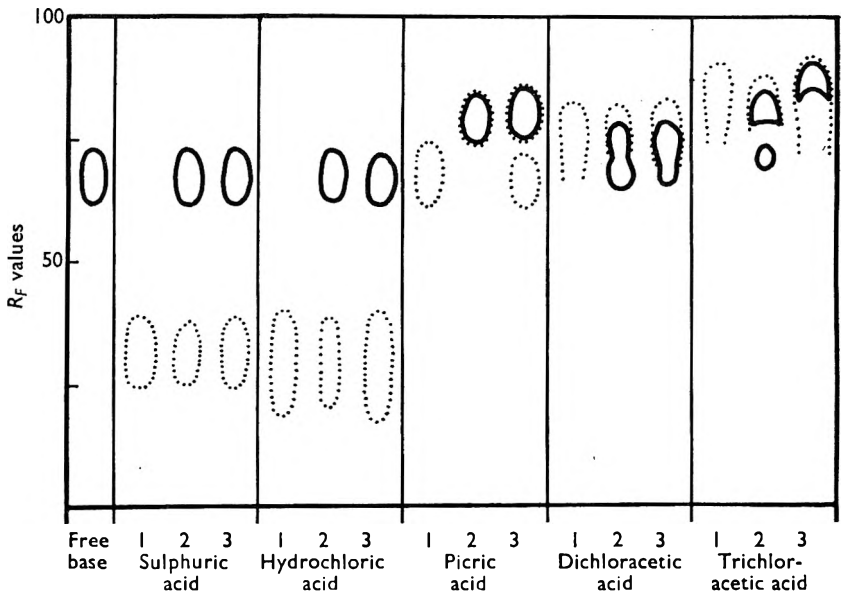

FIG. 3. Chromatograms of β-phenylethylamine developed in the butanol:acetic acid:water solvent system and applied to the paper as a solution (2 μl.) of the free base alone and in the presence of various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

noradrenaline, dichloracetic acid and maleic acid caused isoprenaline to form two amine spots. The trichloracetate and trifluoracetate of isoprenaline yielded results similar to those obtained for these salts of adrenaline and noradrenaline, except that the R_F value of the isoprenaline spot associated with the acid was higher than for the corresponding spots produced by the slower-running amines. Increasing the relative proportion of the acid again increased the amount of the amine present in the faster spot associated with the acid.

Amines having high R_F *values* (70–100), *for example, β-phenylethylamine* (R_F 73 ± 2), *ephedrine* (R_F 75 ± 3) *and amphetamine* (R_F 76 ± 3). Figure 3 summarises the results for this group of substances. These

amines were unaffected by the presence of mineral acids even when the latter were present at a sixty molar excess; the R_F values of the individual components corresponded with those of the free base and acid.

Single amine spots associated with picric acid resulted from β-phenylethylamine, ephedrine and amphetamine picrates (R_F values of 83, 86 and 88 respectively). The R_F values of these spots were higher than those of either of the components; both ephedrine and amphetamine have higher R_F values than picric acid. Any excess picric acid gives the normal spot for the acid (i.e., one having an R_F value of 74) in addition.

Unlike the picrates, β-phenylethylamine, ephedrine and amphetamine maleates yielded a single amine spot associated with the acid having R_F values of 75, 77 and 76 which were not significantly higher than those of the free acids and bases. Excess maleic acid, if present, gave a slower-running spot (R_F 72) with ephedrine and amphetamine.

Two amine spots resulted when the stronger organic acids were present providing that the R_F values of the components differed sufficiently. The R_F value of the base associated with the trichloracetic or trifluoracetic acids was higher than that for the amines of lower R_F value and a greater proportion of the base was associated with the acid in this case.

The weaker acids, for example, tartaric, citric, benzoic or formic, did not affect the running of any of the amines.

Thus, all the strong acids affected the chromatographic behaviour of the amines unless the R_F values of the individual components differed greatly. The R_F value of the amine spot associated with the strong acids of high R_F value is dependent upon the relative values of the individual components: the higher the normal values, the faster the amine–acid spot and the greater the proportion of the base in this spot. The strength of the acid also affects the latter proportion. Acids having pKa values of greater than 3 did not affect the chromatographic behaviour of the amines under the conditions used. When the amine salt is chromatographed, the quantity applied to the paper is important. For example, elongated amine spots were obtained when increasing quantities (5, 7·5, 10, 15 and 20 μl.) of a 0·05M solution of β-phenylethylamine sulphate were applied to the paper and chromatographed; the lengths of the spots were proportional to the amount added and some overlapping of amine and acid spots occurred. However, β-phenylethylamine and the other faster amines were unaffected when 0·1 μM quantities were applied in solutions of increasing mineral acid concentration. Similarly, the amine spot was identical whether 0·1 μM adrenaline was applied as the free base or as the maleate but upward streaking occurred when larger quantities (0·2 to 0·4 μM) of the salt were chromatographed. Tailing was observed, particularly where the R_F values of the amines spots were not widely separated from those of the acids; the spots became more discrete as the amount of the amine applied was decreased, that is detection of tailing is a function of the reagent sensitivity for the amine.

Two-dimensional chromatograms of both isoprenaline and β-phenylethylamine trichloracetates were prepared using the butanol : acetic acid : water solvent system. The results for the isoprenaline salt are shown in

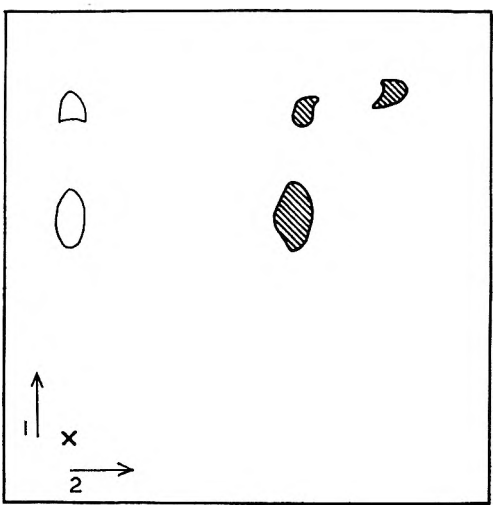

FIG. 4. Two dimensional chromatogram of isoprenaline trichloracetate (4 μl. of 0·05M solution) developed using the butanol: acetic acid: water solvent system. The unshaded spots indicate the positions of the amine after the first run.

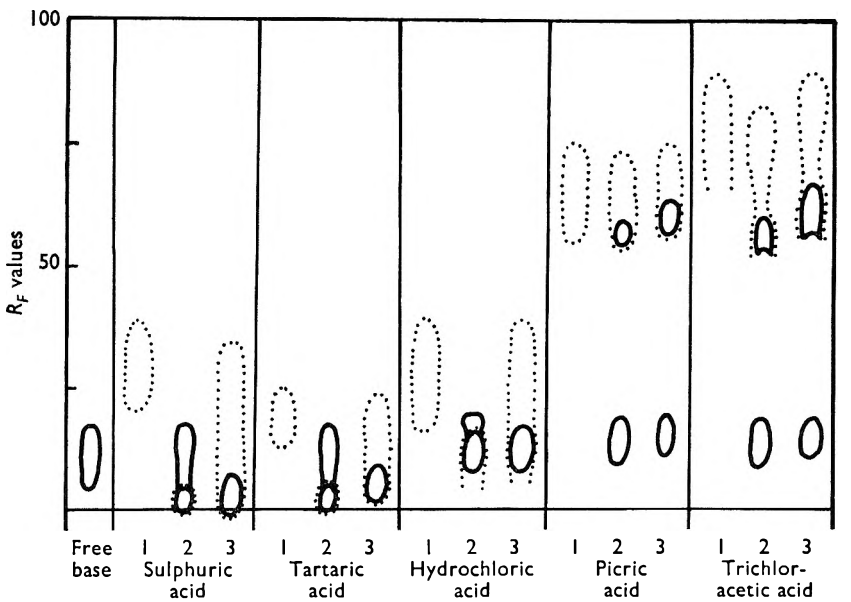

FIG. 5. Chromatograms of adrenaline developed in water-saturated butanol when applied as a suspension (2 μl.) of the free base or as a solution (2 μl.) containing various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

Figure 4 and are similar to those for β-phenylethylamine trichloracetate. The single dimensional chromatogram shows two isoprenaline spots: the faster spot (R_F 82) in which the amine is associated with acid again separates into two on re-chromatographing while the slower free amine spot persists. The shape and relative location of the two new spots in the plane at right angles to the direction of development is characteristic of the location of the base in the parent base–acid spot resulting in the single dimension chromatogram.

Results Using Water-Saturated n-Butanol Solvent

Amines having low R_F *values (less than* 40), *for example, noradrenaline* (R_F 15 ± 2), *adrenaline* (R_F 17 ± 3), *isoprenaline* (R_F 28 ± 4) *and tyramine*

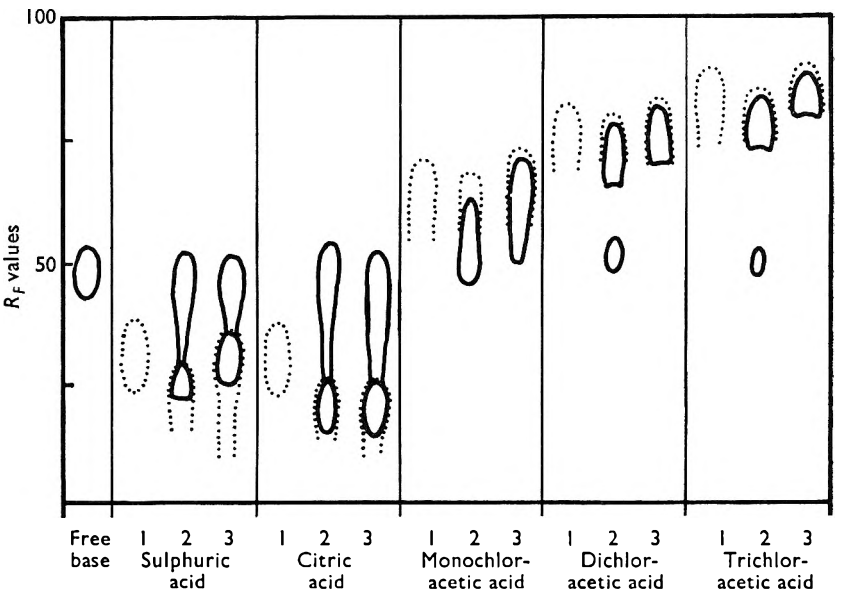

FIG. 6. Chromatograms of β-phenylethylamine developed in water-saturated butanol when applied as a solution (2 μl.) of the free base alone and in the presence of various acids. Continuous outline indicates the amine spot. Dotted outline indicates the acid spot.

1. Free acid (0·5N).
2. Amine plus an equivalent amount of acid (0·05M solution).
3. Amine (0·05M solution) with a tenfold excess of acid.

(R_F 36 ± 3). Both adrenaline and noradrenaline hydrochlorides and sulphates produced two linked spots (see Fig. 5), for example, adrenaline sulphate gave amine spots having R_F values of 4 and 16. An excess of the acid caused localisation of the amine in a single spot having a value of 7. Polybasic organic acids, such as citric and tartaric acids, caused a similar effect. Two amine spots were produced by the *o*-nitro-*p*-hydroxybenzoates, dichloracetates, trichloracetates, trifluoracetates and picrates but not the benzoates or salicylates of the above amines. For example, adrenaline trichloracetate gave amine spots having R_F values of 18 and 60

and adrenaline benzoate gave a single amine spot (R_F 17). Monochloracetic acid only caused elongation of the amine spots.

Amines of moderate R_F *values (greater than* 40), *for example, β-phenylethylamine* (R_F 52 ± 3), *amphetamine* (R_F 60) *and ephedrine* (R_F 56 ± 5). The results are shown in Figure 6. Two linked amine spots were observed

TABLE I

R_F VALUES OF THE AMINES IN THE LINKED AMINE SPOTS OBTAINED WHEN VARIOUS SALTS OF β-PHENYLETHYLAMINE AND EPHEDRINE ARE CHROMATOGRAPHED IN THE WATER: SATURATED BUTANOL SYSTEM

Substance chromatographed	R_F values of the amine spots	
β-Phenylethylamine		
Citrate	23	41
Oxalate	14	51
Hydrochloride	50	57
Sulphate	26	51
Ephedrine		
Citrate	26	50
Sulphate	26	55
Hydrochloride	42	55

when the amines in this group were applied to the paper with an equivalent amount of sulphuric acid or a polybasic organic acid and chromatographed (Table I).

When an excess of any of the acids was used, most of the amine was present in the slower-running compact spot. For example, β-phenylethylamine in excess sulphuric acid gave a dense spot of R_F 38 and a less dense spot of R_F 51.

Multiple spot formation was again observed with the stronger organic acids of high R_F values including dichloracetic, trichloracetic, trifluoracetic, salicylic, 5-nitrosalicylic and *o*-nitrobenzoic acids. Using monochloracetic acid, all the amines gave elongated spots since the values of the

TABLE II

R_F VALUES OF THREE AMINES WHEN APPLIED TO WHATMAN NO. 1 PAPER AS SOLUTIONS IN VARIOUS ACIDS AND DEVELOPED WITH THE n-BUTANOL : AMMONIA : WATER SOLVENT SYSTEM

Acid	β-Phenylethylamine		Ephedrine		Amphetamine	
	Base	Acid	Base	Acid	Base	Acid
Sulphuric	93	4	94	3	94	3
Hydrochloric	94	12	96	11	95	12
Trichloracetic	93	61	95	59	95	60
Picric	93	86	95	78	96	80
Citric	91	4	94	3	—	—
Acetic	93	—	93	—	—	—
Free base	92 ± 2		94 ± 3		95	

acids and bases were not too dissimilar. Figure 6 illustrates the results obtained using β-phenylethylamine and various acids, including the three chloracetic acids, in a neutral solvent system.

Many acids of higher pKa values, except the more volatile formic and acetic acids, affected the chromatographic behaviour of the amines in the neutral solvent system when no effect had been observed with these acids.

in the acidic solvent system. Tailing was less extensive. More amine spots were associated with the acid spots in this system unless the R_F values of the components differed greatly.

Results Using Other Solvents

Results using n-butanol: ammonia: water. None of the acids tested affected the R_F values (see Table II) of the stable sympathomimetic amines (i.e., β-phenethylethylamine, ephedrine and amphetamine). All the bases containing the catechol moiety (i.e., adrenaline, noradrenaline and isoprenaline) decomposed under the conditions used.

TABLE III

R_F VALUES OF SOME SYMPATHOMIMETIC AMINES WHEN APPLIED TO WHATMAN NO. 1 PAPER AS SOLUTIONS IN VARIOUS ACIDS AND DEVELOPED USING LIQUEFIED PHENOL AS THE RUNNING SOLVENT

Acid	Adrenaline		Isoprenaline		β-Phenyl-ethylamine		Ephedrine	
	Base	Acid	Base	Acid	Base	Acid	Base	Acid
Sulphuric	44,† 77	47	82	38	91	38	93	37
Hydrochloric	54,† 75	54	83	50	91	48	93	46
Trichloracetic	82	*	89	*	92	*	94	*
Picric	81	81	82	82	93	93	92	78, 92
Salicylic	88	88	87	87	92	92	93	93
Benzoic	86	*	86	*	—	—	—	—
Citric	41,† 74	*	46,† 82	*	92	*	92	*
Tartaric	45,† 75	*	44,† 82	*	91	*	93	*
Free base	76 ± 3		82 ± 1		92 ± 1		93 ± 2	

† Tailing between two spots.
* Not detected.

Results using Liquefied Phenol. Most acids altered the chromatographic behaviour of the amines unless there was a considerable difference between the R_F values of the two components. The results are summarised in Table III. The slower amines, e.g., adrenaline (R_F 76) and noradrenaline (R_F 71) were affected by several acids, including citric, tartaric, hydrochloric and sulphuric acids.

Discussion

Multiple amine spot formation of sympathomimetic amines on paper chromatograms developed in neutral and acidic solvent systems in the presence of added acids is dependent upon the following major factors:

(1) the relative R_F values of the amines and the added acids;
(2) the relative dissociation constants of the acid present in the running solvent and that applied with the amine;
(3) the relative proportion of the bases and added acids and
(4) the relative local concentrations of the added acid and that present in the solvent system.

Complexing between the acid and base may alter the chromatographic behaviour of the compounds.

When an acid is present in the solution of an amine applied to a paper chromatogram which is then developed with a neutral or acidic solvent system the R_F value of the amine spot may be affected and a pure base may form two spots. If the added acid has a pKa value much less than

that of the solvent system and a normal R_F value greater than that of the free amine, double amine spot formation may be anticipated provided that the R_F values of the two components are neither too close nor too divergent. The faster amine spot will be located as a crescent at the apex of the acid spot and the slower amine spot consists of the free amine in equilbrium with the solvent. The quantity of amine present in the faster spot is dependent upon the strength of the added acid, the relative concentrations of this acid and base and the relative R_F values of the two components: the greater the concentration of acid relative to that of the amine, the greater the proportion of the amine associated with the acid in the faster spot. If sufficient excess of the acid is applied and the R_F values of the acid and base do not differ greatly, all (apparently) of the amine will be associated with acid. When double amine spot formation occurs with organic acids of high R_F values, for example, β-phenylethylamine or tyramine and either trichloracetic or trifluoracetic acids, the R_F value of the amine in the acid spot can be increased by increasing the relative proportion of acid added (see Fig. 3). The stronger the acid the greater will be the proportion of amine held in the faster spot but, with a given acid, the greater the difference between the R_F values of the free base and acid, the smaller will be the proportion of amine associated with the faster amine–acid spot. A single amine spot associated with acid is obtained when the R_F values of the two components are too close and complete separation of amine (to form one spot) and acid spots will occur when their R_F values are too divergent.

Chromatograms of amine salts (acidic solvent) in which the acid component has a lower R_F value than the amine show elongated amine spots associated with the acid, except with fast-running amines when complete separation of the acid and amine occurs. In a neutral solvent system the amine forms two linked spots, the slower of which is associated with acid: the amine–acid spot has a lower R_F value than either of the components (see Fig. 5). The slower-running amines, e.g., noradrenaline and adrenaline, will also form "spanner" or "inverted-crescent" shaped spots in the presence of an excess of a polybasic mineral acid. This may be attributed to the differing R_F values of the mono-, di- and tribasic salts of the acids.

The strength of the added acid is also important. Although the R_F value of free dichloracetic acid is closer to that of adrenaline than is that of trichloracetic acid, the presence of an equivalent amount of the latter acid (pKa 0·65) will cause formation of two amine spots, whereas, the weaker dichloracetic acid (pKa 1·29) does not produce this effect (see Fig. 1). The range of R_F values within which amines are affected by a given acid (the R_F values of the two components being not too dissimilar) is dependent upon the pKa value of that acid and to a lesser extent upon the relative concentrations of acid and base. Amines chromatographed in a neutral solvent system are affected by many weak acids.

Complex formation may also play a role in multiple spot formation of amines since some amine picrates produce an amine–acid spot having a higher R_F value than either of the components (see Fig. 3).

The use of two-dimensional chromatography with amine salts using the same solvent system yields interesting information. If two spots are formed in unidimensional chromatography, for example, isoprenaline trichloracetate (see Fig. 4), then the free amine spot will run as an entity in the second dimension and its centre will be on the line drawn in the direction of development through the centre of the parent spot; the amine associated with the acid will separate into two spots, one of free amine and one of amine associated with acid, and the shape of these spots is predictable. For example, if the acid runs faster than the amine and the amine associated with the acid is localised towards the bottom of the acid spot, then in the second development the new base spot associated with the acid will be beyond and above that of the new base spot (considering the first development to have been in the vertical plane) and will have a lower trailing tail while the new free base spot will have an upper leading horn (see Fig. 4). This situation obtains because, after the first development, the main portion of acid is located as free acid above the base associated with it so that in the second development more facile separation of the base as free base occurs from the lower portion of the base–acid spot than from the upper portion. If the acid has a lower R_F value than that of the base and two spots result, then the upper spot will run as an entity in the second dimension while the lower one will form two spots during the second development, the leading one being free base and located above the slower amine–acid spot. If an amine salt (using unidimensional chromatography) yields one amine spot completely separated from the acid component the second development will again yield a single amine spot. If a single amine–acid spot faster than either component is obtained (a complex), then the second development will again yield a single spot of amine associated with the acid spot.

The practice of applying solutions of known substances in water, acid or any other solvent to produce a reference paper chromatogram may produce misleading information if the reference chromatograms are to be compared with those of biological fluids or extracts. For instance, if during the investigation of the metabolism of an amine drug four amine spots are observed upon chromatography of urine, it does not necessarily indicate that three metabolites of the parent drug are present if one of the spots observed with the urine sample is identical with that obtained from a reference chromatogram prepared by applying a solution of the pure drug in one of the components of the solvent system. For example, pure β-phenylethylamine or pure ephedrine dissolved in urine and chromatographed using the neutral solvent system gave several amine spots; the control solution in water produced a single spot and the urine alone under the conditions used did not give any spots of R_F values similar to those produced on addition of the amines to the urine.

Various methods may be used to overcome this problem. The simplest solution would be to render the biological fluid alkaline and extract the base from the interfering acids and apply the base to the paper as a solution in one of the components of the solvent system used for chromatography. This would not be satisfactory if an acidic grouping were also

present in the molecule or if the materials were unstable to alkali. Difficulty may also occur if trichloracetic acid has been used as a protein precipitant during the preparation of the biological extract for paper chromatography, because in spite of repeated washings of the organic solvent with water, traces of the acid sufficient to affect the chromatographic behaviour of the base may be present; this effect is more serious if the solutions are made alkaline with ammonia solution rather than with sodium hydroxide solution since the partition of the trichloracetic acid into the organic phase is more favoured when ammonia is present. In these circumstances two dimensional chromatography using the same solvent may yield useful information. A spot which consists of free amine will still run as a single spot on the second dimension whereas most amine–acid spots of the first dimension will separate into two spots in the second dimension; one of these spots, and the single free amine spot, will be equi-distant from the solvent front of the second development. The use of different solvent systems is recommended, for example, if the reference compound in the biological fluid after chromatography gave more than one spot in a neutral solvent system the use of an acidic and basic solvent system is recommended. If more than one base spot is obtained using the biological extract, then it is advisable to use a control by adding the substance suspected of being present to an aliquot of the biological fluid. An increased intensity of one or more of the spots will be observed but no additional spot should appear if the added material is present in the biological extract. Further reference chromatograms obtained by adding the suspected compound to an aliquot of the biological fluid from which basic substances have been extracted is also useful: if the extracted biological fluid gives no spots upon chromatography but gives more than one spot upon the addition of the reference substance then the interference of acids may be suspected. The addition of another base substance of slightly differing R_F value to that of the suspected substance to an aliquot of the extracted biological fluid will yield one base spot of slightly different R_F value from that of the suspected compound, but the spots resulting from the association of the base with the acid will be identical with those obtained using the suspected compound in the biological extract, for example, β-phenylethylamine and ephedrine in urine samples. If metabolites of the parent drug are present, then additional spot(s) will be present in the test chromatogram when compared with the reference chromatogram obtained by applying the drug as a solution in the biological fluid tested.

Acknowledgement. One of us (M.A.B.) is indebted to the Pharmaceutical Society for an educational grant.

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After Mr. Beaven presented the paper there was a DISCUSSION.

THE ASSAY OF STILBOESTROL BY THE ISOTOPE DILUTION TECHNIQUE

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The isotope dilution technique has often been used for analysing complex mixtures. In principle a small amount of radioactive compound which is isotopic with and in the same chemical form as the compound to be determined is added to the mixture of organic compounds. A pure derivative, is then isolated not necessarily completely, and its radioactivity measured. From the change in radioactivity per unit weight, specific activity, it is possible to calculate the quantity of compound present in the original mixture.

To a sample of stilboestrol was added ^{14}C labelled stilboestrol of known specific activity and the mixture was acetylated according to the B.P. 1958. The specific activity of a portion of the acetylated derivative was then determined, and from the change in activity the purity of the stilboestrol was calculated.

The measurement of ^{14}C ($\beta = 0{\cdot}155$ MeV) presents difficulty because of the low energy of the β-particle emitted, and it is only recently that the liquid scintillation technique has been introduced as a method of counting low energy β-particles. In this technique the sample containing the radioactive substance is added to a solution of the phosphor and the light flashes emitted by this mixture of substances dissolved in an organic solvent, when irradiated, are detected and amplified by a photomultiplier and then counted.

Davidson and Feigelson[1], and Bell and Hayes[6], have reviewed the applications and instrumentation of liquid scintillation counting respectively, and Stitch[2] has determined the variables affecting the method with particular reference to ^{14}C steroids. One advantage over previous methods is that the fixed 4Π geometry makes the method highly sensitive and reproducible.

Description of the Apparatus

The phosphor consisted[3] of naphthalene 80 g., diphenyloxazole (P.P.O.) 5 g., 1,4-di-2-(5-phenyloxazolyl)benzene 0·05 g., xylene 390 ml., 1,4-dioxane 390 ml., ethanol 235 ml. The reagent grade naphthalene was recrystallised from 80 per cent ethanol. The xylene and dioxane were refluxed with, and distilled from sodium. The absolute ethanol was dried by a method of Vogel[4]. The mixed solute was dissolved in 700 ml. of solvent and made up to 1 litre with additional solvent and was stored in a dark brown bottle in the refrigerator.

The photomultiplier tube (E.M.I. 6097s) was selected because of its low dark current and housed in a lead castle designed for liquid scintillation counting (Panax SC/LP). The castle was maintained at 5° to reduce the dark current to a minimum. The radioactive sample was added to the phosphor which was contained in a counting jar. The jar was immersed in silicone oil (MS200/20) to ensure a good light path between the bottom of the jar and the top of the photomultiplier tube. No air bubbles were allowed beneath the jar as this impairs the efficiency of the counter. When the castle door was closed a shutter mechanism between the counting jar and the photomultiplier tube opened and allowed light flashes to reach the photomultiplier. The pulses obtained were amplified with a Panax 4250 low gain amplifier (× 1,000) and then counted with a Panax D657C scaler operated with a Panax T300 timer. The optimum working conditions were found to be 1,450 volts with the discriminator set at 12 volts. The resolution time of the first dekatron tube (GC10D) was 50 microseconds.

TABLE I

Reference sample counts sec^{-1} corrected for lost counts and background A	Weight of stilboestrol g. X	Weight of acetylated stilboestrol g. W	Count rate of acetylated sample counts sec^{-1} corrected for lost counts and background B	Stilboestrol per cent $= \frac{A}{B} \times \frac{W}{X} \times 100 \times \frac{268{\cdot}4}{352{\cdot}4}$
1. 6429*	1·4771	0·4550	1,486	101·5
2. 3144	1·5000	0·1162	187	99·2
3. 3253	1·7510	0·0989	141	99·2
4. 3419	1·6308	0·2100	344	97·5

* 0·060 ml. of active stilboestrol solution.

Assay details. The ^{14}C labelled stilboestrol (Radiochemical Centre, Amersham) was dissolved in ethanol and its specific activity was approximately 2μc per ml.

The background count per 100 seconds with the phosphor alone in the jar was first determined. To the phosphor was then added 0·030 ml. (= 5μg.) of active stilboestrol solution using an "Agla" syringe, and the count rate was redetermined giving the rate of the reference solution.

Approximately 1·5 g. of Stilboestrol B.P. (Ward Blenkinsop) and 0·030 ml. of active stilboestrol solution were acetylated according to the B.P. method[5]. The acetylated stilboestrol was filtered on a No. 3 sintered glass filter and then dried at 110° for 1 hour. Approximately 100 mg. of this precipitate, accurately weighed was added to a sample jar containing phosphor and the count rate per 100 seconds determined. The acetylated stilboestrol was very soluble in the phosphor and amounts up to 520 mg. added to 12 ml. of phosphor (capacity of the counting jar) did not quench the light pulse. The results are shown in Table I.

The preliminary results show that the method should be useful for the assay of steroids which do not acetylate quantitatively, provided that the acetylated derivative (or any other suitable derivative) can be obtained in a pure form, for example, by repeated recrystallisation. The method suffers from the same limitations as the B.P. assay of stilboestrol in that up to 5 per cent monoacetylmonomethyl stilboestrol will pass undetected.

The author wishes to thank Mr. D. Russell who did much of the practical work.

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After the Author presented the paper there was a DISCUSSION.

THE DETERMINATION OF n-VALUES FOR SOME AMINOACRIDINES BY CONTROLLED POTENTIAL COULOMETRIC REDUCTION

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Received May 20, 1960

The reduction of some aminoacridines has been investigated by controlled potential coulometry. The number of electrons involved in the first reduction stage has been determined by a graphical treatment of the current-time values obtained using a divided electrolysis cell. With the exception of 5-aminoacridine the results confirm the values obtained indirectly by Kaye[5].

The method of controlled potential electro-reduction at a stirred mercury cathode was introduced by Lingane and is discussed in his paper[1] and in his book[2]. This procedure was developed primarily as an analytical method but it can also be adapted to determine the number of electrons involved (n-value) in the reduction process at the cathode[1].

Kaye and Stonehill[3] in their investigations of the polarographic reduction of some aminoacridines concluded that at the dropping mercury electrode the reductions take place in two one-electron stages, with the intermediate formation of a stable semiquinone radical. The n-value they obtained was deduced by an application of the Ilkovic equation[4], making assumptions about the value of the diffusion coefficient of the aminoacridine in ethanol.

Since Kaye's theory of the antibacterial action of the aminoacridines[5] is based on this interpretation, it was thought to be of importance to determine the value of n by an independent method.

Methods

The circuit and apparatus described by Lingane[1,6] was used in preliminary experiments. In these a controlled potential, obtained from a potential divider, was applied to the electrolysis cell, consisting of a silver–silver chloride anode and a mechanically stirred mercury cathode of about 30 sq. cm. surface area. The potential of the cathode was measured with reference to a saturated calomel electrode and the applied voltage was adjusted manually so as to maintain this potential at an appropriate value. The quantity of electricity consumed in the reduction of a known amount of oxidant was measured by means of a hydrogen-oxygen gas coulometer in series with the reduction cell. The coulometer contained potassium sulphate (0·5M) as electrolyte. The corrected volume of mixed gas per coulomb was determined and its value found to agree with that obtained by Lingane[1].

According to Lingane [1,6] the current should decrease exponentially with time during the course of the reduction (equation 1).

$$I = I_0 10^{-k't} \qquad (1)$$

where I is the current measured at time t, I_0 is the initial current and k′ is a constant depending on the cell geometry, rate of stirring and temperature. However when 5-aminoacridine was reduced in this cell the current fell to a constant value of approximately half the initial value and a graph of log I against time was not linear, indicating that the fall was not in accordance with equation 1 (Fig. 1).

It was considered that this was due to reoxidation of the reduced material at the anode. To eliminate this possibility a divided cell was constructed, similar to that described by Lingane (ref. [2], p. 478), in which

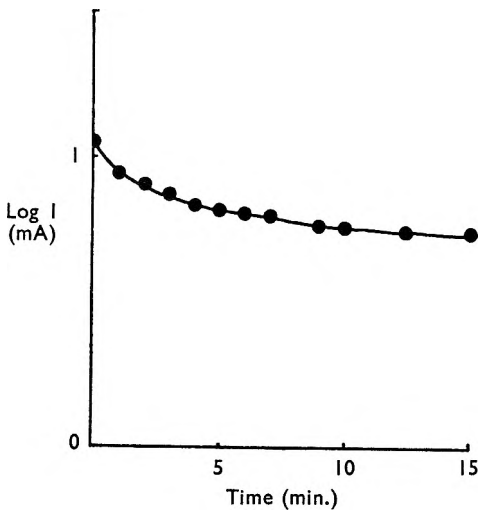

FIG. 1. Decay of current with time (undivided cell) 100 mg. of 5-aminoacridine in alcoholic buffer. 1300 mV *v* SCE.

anolyte and catholyte are separated by a sintered glass disc covered with an agar plug. On repeating the reduction of 5-aminoacridine in this cell, at a potential of 1,300 mV *versus* a saturated calomel electrode, corresponding to the top of the first step of the polarographic wave of 5-aminoacridine[3], the current was observed to fall exponentially as required by equation 1, and the calculated n-value was found to be approximately two.

When 2,8-diaminoacridine was examined in a similar manner, n-values of 0·81, 1·0, and 1·08 were found.

In all these experiments the precise control of potential caused difficulty, and in addition each reduction was time consuming (90–120 min.). In the early stages of each reduction it was found that the desired potential could not be achieved, due to the variability of the voltage drop across the gas coulometer. Any increase of the applied voltage beyond a certain point had little effect on the potential of the stirred cathode. On shorting out the coulometer the desired potential could be achieved without difficulty.

MacNevin and Baker[7] have pointed out that integration of equation 1 will yield the necessary quantity of electricity and experiments were

made with the object of investigating the regularity with which the current decayed, with a view to using a graphical method of current integration. These revealed that stirring rate was the only important factor influencing the rate of a given reduction (i.e., the value of k′ in equation 1). Stirring by means of paddle proved to be too irregular to give a good linear graph of log I against time. On replacing the paddle by a slotted polythene disc with a flat sided polythene covered magnetic paddle fused to it, the current was found to decay smoothly with time provided the stirring rate was not excessive (Fig. 2).

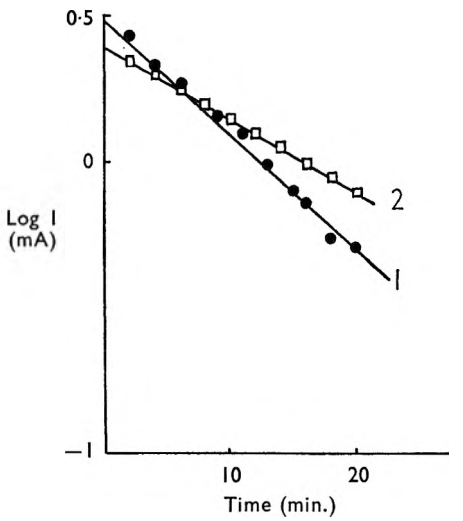

FIG. 2. Decay of current with time (divided cell), 5 mg. proflavine base in alcoholic buffer, 1200 mV *v* SCE. 1, rapid stirring; 2, moderate stirring.

For convenience in evaluation the concept of "half time" of the reaction was used.

$$\text{Since } I = I_0e^{-kt} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

when $t = t_{\frac{1}{2}}$ (the time required for the current to fall to half of its original value, i.e., the time required to reduce half of the oxidant), then

$$\tfrac{1}{2} = e^{-kt_{\frac{1}{2}}} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

$$\text{when } k = 0{\cdot}693/t_{\frac{1}{2}} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

For half reduction the quantity of electricity required, $Q_{\frac{1}{2}}$, is given by

$$Q_{\frac{1}{2}} = \int_0^{t_{\frac{1}{2}}} Idt = \int_0^{t_{\frac{1}{2}}} I_0e^{-kt}dt$$
$$= (I_0e^{-kt_{\frac{1}{2}}})/k$$

and introducing (3) and (4)

$$Q_{\frac{1}{2}} = I_0t_{\frac{1}{2}}/0{\cdot}693 \times 2 \quad .. \quad .. \quad .. \quad .. \quad .. \quad (5)$$

For reduction of the whole of the sample

$$Q = I_0t_{\frac{1}{2}}/0{\cdot}693 \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (6)$$

When t is in seconds and I is in amperes Q is in coulombs, hence from Faraday's Laws,

$$n = \frac{I_0 t_{\frac{1}{2}} M}{0{\cdot}693 \times 96{,}500 \times w} \quad .. \quad .. \quad .. \quad (7)$$

when w is the weight in g. of reducible substance of molecular weight M.

From the graph of log I against t, obtained by reading I at 1 minute intervals, I_0 and $t_{\frac{1}{2}}$ can be determined. The reduction period was extended to cover about two half times. The circuit and apparatus used for this work are shown in Figure 3.

A Pye "Scalamp" galvanometer was used for current measurement. It was suitably shunted so that 1 cm. deflection corresponded to 1 mA on

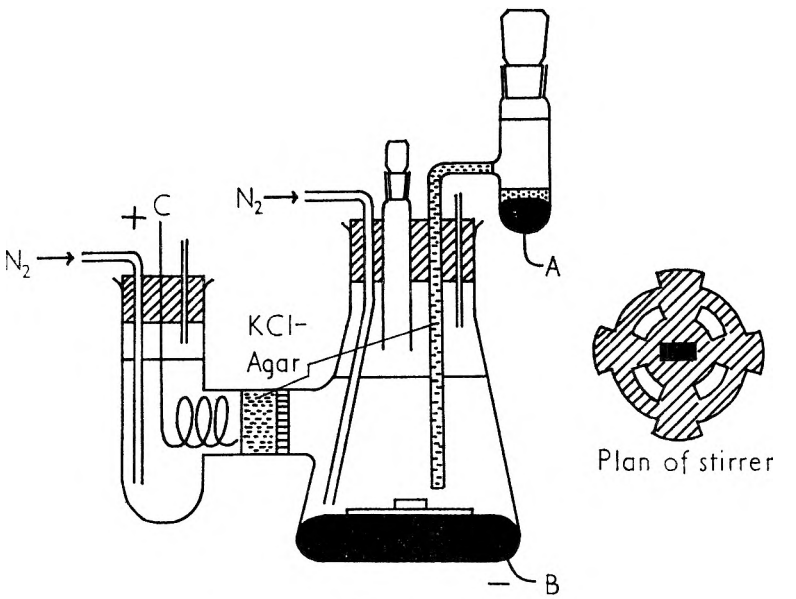

FIG. 3, *a*. Divided cell assembly.

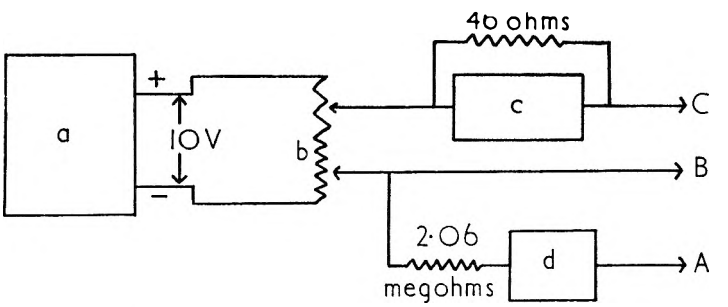

FIG. 3, *b*. Circuit diagram. a, direct current source (Labgear Eliminac); b, potential divider; c, galvanometer for current measurement; d, galvanometer for potential measurement.

the 0·001 range. A Cambridge "Spot" galvanometer was used for measurement of potential, by placing in series a suitable resistance. The current required to give a full-scale deflection did not exceed 1μA, which was too small to affect materially the calculated n-value, and did not alter the potential of the large capacity calomel electrode from that measured with a valve voltmeter.

The solvents used in this work were those which Kaye and Stonehill[3] found to give satisfactory polarographic waves for aminoacridines. Reducible impurities were first removed from the solvent by subjecting 150 ml. to the working potential in the reduction cell in the presence of a stream of oxygen-free nitrogen until the current fell to a constant value of

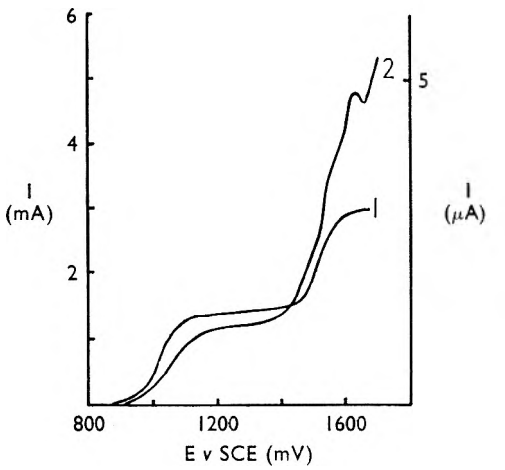

FIG. 4. 1, Polarogram of 2 × 10^{-4} M proflavine base in alcoholic buffer (microamp scale). 2, Current voltage curve at stirred mercury cathode for 1 × 10^{-4} M proflavine base in the same alcoholic buffer (milliamp scale).

0·03 to 0·05 mA. It was assumed that this "background" current remained constant throughout the subsequent reduction, and its value was subtracted from the observed total current before plotting graphically. A full discussion of background corrections has been given by Meites and Moros[8].

It has been pointed out[1,6] that the polarographic method forms a reliable pilot technique to establish the optimum conditions of potential, composition of supporting electrolyte and concentration, for coulometric analysis. In this work however, to establish the optimum value of the working potential, current–voltage curves for the electroreduction of the aminoacridines were obtained by dissolving about 1 mg. of the substance in 150 ml. of reduced ethanolic buffer and measuring the current at 40 mV intervals of potential. This procedure was completed as rapidly as possible to minimise the effect of concentration changes produced by the electrolysis. The current-voltage curve so obtained for 2,8-diaminoacridine is shown in Figure 4, together with a polarogram of the same solution. These curves show that a more negative potential is

required to attain the top of the first step, than is indicated by the polarogram. This was found to be general for the aminoacridines.

As a further precaution a series of current-voltage curves was prepared using quntities of aminoacridine ranging from 1 to 10 mg. Such a series is shown in Figure 5 and it is clear that with increasing concentration definition of the working potential becomes more difficult. Coulometric determinations were therefore made using samples of aminoacridine not greater than 5 mg.

In each experiment 1–5 mg. of the aminoacridine was dissolved in the prepared buffer solution. The control potential was applied and a stop

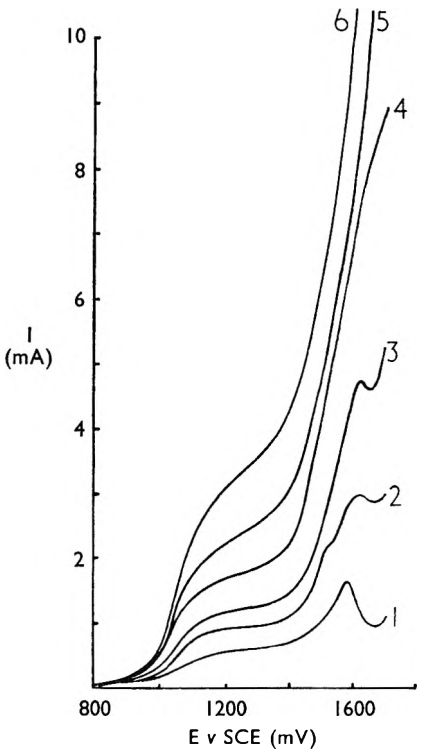

FIG. 5. Current voltage curves for proflavine base in alcoholic buffer (obtained by successive addition of 1, 1, 1, 2, 2 and 3 mg. to a total of 10 mg.).

clock was started simultaneously. Readings of the current were made at 1 minute intervals, the potential being maintained throughout by manual adjustment of the potential divider. Readings were taken until the current had fallen to about one-quarter of its original value (20–30 min.). The results were plotted on semi-log paper. Although replicate determinations on similar samples did not always yield the same values of initial current and half time (Fig. 2), the product of these two factors (see eqn. 6) remained fairly constant.

The quantities of 1-aminoacridine, 2-aminoacridine, 3-aminoacridine and 4-aminoacridine available did not permit more than three or four

determinations to be made, but some indication of the precision of the method is given by the series of n-values obtained for 2,8-diaminoacridine. It is probably not better than ± 10 per cent. However since the object of the present work was to evaluate n-values it was considered that this accuracy was quite adequate when n is one or two.

Results

A summary of these is given in Table I.

TABLE I

SUMMARY OF EXPERIMENTAL CONDITIONS AND RESULTS OBTAINED FOR AMINOACRIDINES

Substance	Potential mV	wt. mg.	M.wt.	I_o mA	$t_{\frac{1}{2}}$ min.	n-value
1-aminoacridine	1,075	5·0	194	3·00	10·5	1·10
"	1,075	5·0	194	3·60	8·7	1·09
2-aminoacridine	1,040	2·4	194	1·35	10·7	1·10
"	1,040	1·14	194	0·70	9·8	1·05
3-aminoacridine	1,020	1·2	194	0·58	11·0	0·93
"	1,020	1·2	194	0·72	10·3	1·08
4-aminocridine	1,100	1·05	194	0·52	10·8	0·93
"	1,100	0·95	194	0·54	10·0	0·99
5-aminoacridine hydrochloride	1,350	0·98	230·5	0·53	19·0	2·13
	1,350	4·6	230·5	1·92	23·0	1·98
2,7-diaminoacridine	1,000	5·0	209	1·55	16·8	0·98
"	1,000	4·8	209	2·32	11·2	1·0
2,8-diaminoacridine	1,200	4·0	209	1·71	11·4	0·91
"	1,200	3·12	209	1·60	10·9	1·04
"	1,200	4·0	209	1·82	11·1	0·95
"	1,200	5·1	209	2·27	11·5	0·96
"	1,200	5·0	209	3·20	7·6	0·91
"	1,200	5·2	209	2·48	11·9	1·06
"	1,200	5·0	209	4·12	6·6	1·02

Discussion

Two conclusions emerge from the results. With the exception of 5-aminoacridine, the first step of the current-voltage curve, which corresponds to the first step of the polarographic wave of the aminoacridines, is shown to involve a one-electron reduction. This is an agreement with the results obtained by Kaye and Stonehill[3]. This observation, is of great importance in relation to the free-radical mechanism of bacteriostasis by the aminoacridines put forward by Kaye[5]. With 5-aminoacridine the first step of the wave appears to correspond to a two electron reduction, in contradiction to Kaye's polarographically determined value of one electron. However the value of the initial current does not differ substantially from that observed with the remaining aminoacridines, and the different result is due to a longer half-time with 5-aminoacridine. It may therefore be the case that the higher result with this substance arises as a result of secondary processes, possibly of the nature discussed by Geske and Bard[9]. This possibility is being investigated.

Acknowledgement. The authors wish to thank Dr. R. C. Kaye for samples of aminoacridines and for helpful discussions and encouragement.

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9. Geske and Bard, *J. phys. Chem.*, 1959, **63**, 1057.

After Mr. Butler presented the paper there was a DISCUSSION.

THE DETERMINATION OF THE RELATIVE CONFIGURATION OF MORPHINE, LEVORPHANOL AND *LAEVO*-PHENAZOCINE BY STEREOSELECTIVE ADSORBENTS

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Received May 23, 1960

The preparation of levorphanol, dextrorphan and morphine stereoselective adsorbents is described. These adsorbents are used to show that the *laevo* isomers of morphine, phenazocine and a related compound, and racemorphan and related morphinans have similar configurations; the analgesic activity of the various compounds resides substantially in these *laevo* isomers.

It has been shown previously that in analgesics of the methadone-type possessing one asymmetric carbon atom, the more active analgesic of each enantiomorphic pair possessed the same configuration[1–3]. This and related information has led to the delineation of analgesic receptor sites of demanding steric requirements[3,4]; the known analgesics have been shown to possess configurations and conformations suitable for association with these sites[3–5].

The analgesic activity of morphine, the morphinans and the benzomorphans has been shown to reside chiefly in the *laevo* isomers of each enantiomorphic pair (see Table I). These structures possess a surface which is complementary to the previously delineated receptor sites; the more analgesically active enantiomorphs would therefore be expected to have the same configurations if the analgesic receptor hypothesis is valid.

The relative configuration of the isomers of the three classes of compounds of Table I has not hitherto been established. Unequivocal chemical methods of synthesis or degradation to produce the evidence for these stereochemical relationships are not currently available. The application of stereoselective adsorbents[10] to the establishing of the stereochemical relationships was therefore investigated.

Experimental

Materials

Sodium silicate solution. Commercial grade material was used—I.C.I. Q.79 containing 8·8 per cent by weight Na_2O; 29·0 per cent by weight SiO_2. Mol. Ratio = 3·4. Sp.gr. at 20° = 1·395.

Levorphanol tartrate. (−)-3-Hydroxy-*N*-methylmorphinan tartrate. $[\alpha]_D^{20} = -14{\cdot}6°$ (C = 3, H_2O) (lit.[12] = −13·8°). Log ϵ = 3·3 at λmax 279 mμ in 5 per cent acetic acid.

Dextrorphan tartrate. (+)-3-Hydroxy-*N*-methylmorphinan tartrate. Log ϵ = 3·3 at λmax 279 mμ in 5 per cent acetic acid.

Benzomorphans. (−)-2′-*Hydroxy*-2,5,9-*trimethyl*-6,7-*benzomorphan.* M.p. 183·5° (lit.[9] 183–184·5°). Log ϵ = 3·29 at λmax 279 mμ in 5 per cent acetic acid.

(+)-2′-*Hydroxy*-2,5,9-*trimethyl*-6,7-*benzomorphan.* M.p. 183° (lit.[9] 183–184·5°). Log ϵ = 3·29 at λmax 279 mμ in 5 per cent acetic acid.

Morphine hydrochloride. M.p. 200° (decomp.) (lit.[13] 200° decomp.). Log ϵ = 3·18 at λmax 285 mμ in 5 per cent acetic acid.

DETERMINATION OF RELATIVE CONFIGURATION

Absorption Measurements

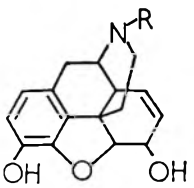
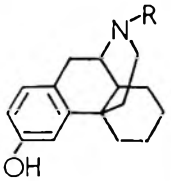
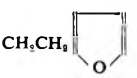
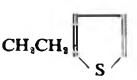
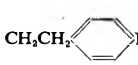
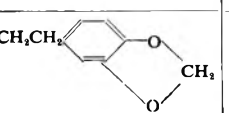
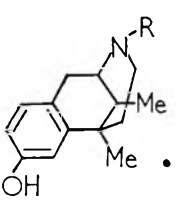
These were made using matched 1 cm. fused silica cuvettes and a Unicam S.P.500 spectrophotometer.

Centrifuge. A "Bara" Gyro Centrifuge was used at 8,500 *g*.

TABLE I

ANALGESIC ACTIVITIES OF ENANTIOMORPHIC PAIRS OF COMPOUNDS OF MORPHINE, MORPHINANS AND BENZOMORPHANS

The recorded analgesic activities are suitable for comparison since they are all obtained by hot plate methods using mice after subcutaneous injection.

Analgesic structure	R	Salt	Isomer	Analgesic activity ED50 mg./kg.	Reference
 (I) Morphine	M	Sulphate	−	10·5	6
			+	almost inactive	7
	Me	Tartrate	−	0·3	8
		HBr	+	44·3	
	CH_2CH_2Ph	HBr	−	0·113	8
		HBr	+	> 100	
 (II) Morphinans		HCl	−	0·010	8
		HCl	+	> 100	
		HCl	−	0·019	8
		HCl	+	> 100	
		Base	−	0·018	8
		Base	+	> 100	
		HCl	−	0·065	8
		HCl	+	> 100	
 (III) Benzomorphans	Me	Base	−	1·7	9
		Base	+	> 20	
	CH_2CH_2Ph	HBr	−	0·11	9
		HBr	+	6·7	

METHODS

Preparation of Adsorbents

Sodium silicate (42 g.) was made up to 200 ml. with distilled water in which 0·5 g. of the reference compound had been dissolved—vigorous stirring being maintained throughout the addition. Approximately 5·7N HCl (130 ml.) was immediately added with continued vigorous stirring. The resultant solution set to a firm gel in 24 hours and the gel was allowed to stand at room temperature for a further 6 days. It was then broken up, spread on sheets of filter paper and allowed to dry in a fume cupboard for 7 days (at this stage the gel was yellow in colour). The gel was subjected to Soxhlet extraction with methanol until colourless, and was then allowed to dry overnight.

The gel was ground in a glass mortar and sieved—those particles between 60 and 200 mesh being retained. These particles were re-extracted with methanol until such time as the reference compound was shown to have been "completely" extracted. The gel was then allowed to dry overnight, sieved to remove any particles of less than 200 mesh which had been formed during the second extraction period and was then ready for use.

A control adsorbent was prepared in exactly the same way but omitting the reference compound. Adsorbents were prepared in this way in the presence of morphine hydrochloride, levorphanol tartrate and dextrorphan tartrate.

Notes on Adsorbent Preparation

1. *Stirring.* Vigorous stirring must be maintained throughout the addition of the acid since inefficient stirring, or slow addition of the acid, was found to cause premature precipitation which produced clumping of the gel.

2. *Drying.* During drying, the filter paper was supported on polythene sheets stretched on wooden frames and supported on a wooden rack. (The acid fumes evolved during drying caused such corrosion of metal frames as to expose the gel to possible contamination). To facilitate drying with the minimum of dust contamination, the frame was placed in a fume cupboard, the air inlet of which was covered by a sheet of muslin.

3. *Preliminary extraction.* At the end of the drying period, the gel was extremely acidic and covered with minute crystals of sodium chloride which gave it a "floury" appearance. The preliminary extraction (*a*) removed the sodium chloride crystals from the surface of the gel, (*b*) reduced the high acidity of the gel (otherwise the gel attacked the metal sieves which were used) and (*c*) left the gel in a more brittle condition, thus facilitating grinding.

4. *Sieving.* To obtain the maximum yield of suitable adsorbent, it was found better to perform numerous sieving and grinding operations rather than reducing the gel to a comparatively fine powder in one operation.

5. *Testing for "complete" extraction.* Complete extraction of the reference compound is never achieved in the true sense as some reference material remains trapped inside the particles of adsorbent; the term is used here to indicate that all the material accessible to the methanol had been removed. In testing for "complete" extraction, methanol (approximately 10 ml.) was removed from the Soxhlet containing the stereoselective adsorbent, evaporated to dryness and the residue, if any, taken up in 5 per cent acetic acid (10 ml.). The resultant solution was centrifuged for 10 minutes and, using in the reference cell a solution prepared

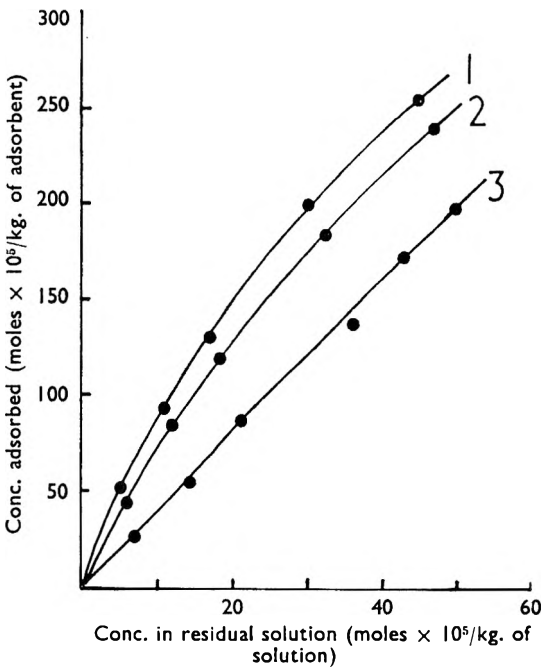


FIG. 1. Adsorption of levorphanol and dextrorphan on levorphanol-selective and control adsorbents. (1) Levorphanol and (2) dextrorphan on levorphanol-selective adsorbent. (3) Levorphanol and dextrorphan on control adsorbent.

similarly from the methanol in contact with the corresponding control adsorbent, spectrophotometric readings were taken in the region of the light absorption peak of the reference compound. Solutions prepared in distilled water were tested for absence of chloride ion.

6. *Ageing of adsorbents.* In general, adsorption measurements were carried out within 2 or 3 weeks of the preparation of the adsorbents.

Measurement of Adsorption

A sample (exactly 1 g.) of the adsorbent (either stereoselective or control), was weighed into a tared glass tube (2·5 × 10 cm.) fitted with

a ground glass stopper. The adsorbent was then washed by rapid shaking with 3 × 10 ml. of a solution of 5 per cent acetic acid, over a period of 1 hour. After shaking, the adsorbent was allowed to settle, and as much as possible of the acid removed without disturbing the adsorbent. This was achieved by using a fine suction tube, the end of which was bent so as to be parallel to the surface of the liquid.

After this preliminary washing, a further 10 ml. of acetic acid solution was added and the adsorbent shaken for a further 1 hour. A portion of the acid was then removed, centrifuged, and absorption measurements taken around the peaks for the compound which was going to be adsorbed

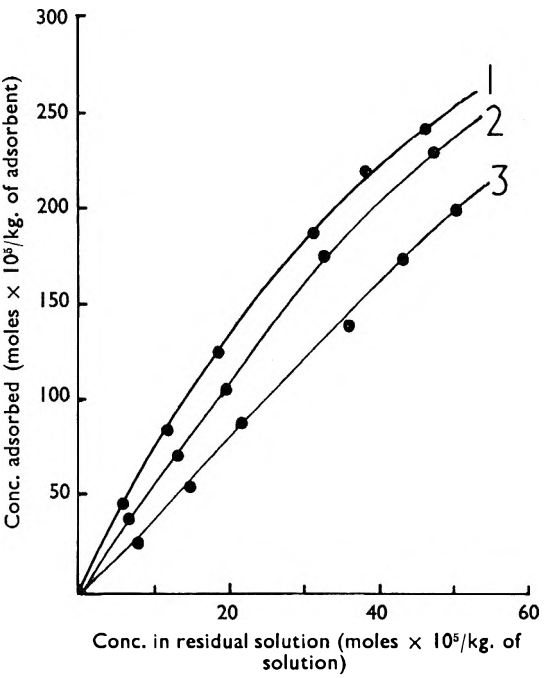


FIG. 2. Adsorption of dextrorphan and levorphanol on dextrorphan-selective adsorbent and control adsorbent. (1) Dextrorphan and (2) levorphanol on dextrorphan-selective adsorbent. (3) Dextrorphan and levorphanol on control adsorbent.

and for the reference compound. In general, zero readings were obtained, but if not, the washing procedure was continued until zero readings were obtained.

After the final washing, as much of the acid solution as possible was removed, and the weight adjusted with 5 per cent acetic acid to that amount which, when the compound to be adsorbed (in 5 per cent acetic acid) was added, it gave the required starting concentration, and a solution to adsorbent ratio of 10 : 1 by weight. The adsorbent and solution were then shaken for 1 hour, by which time equilibrium was reached in these systems.

After 1 hour, the adsorbent was allowed to settle, the supernatant liquid decanted off and centrifuged for 10 minutes. The centrifugate was pipetted off and its concentration determined spectrophotometrically.

By the above method, duplicate readings for a particular adsorbent in the same experiment using separate tubes and the same starting concentration of adsorbate, showed good agreement, e.g. 94 of 109 pairs of readings agreed to within 3 per cent for the amount of material adsorbed.

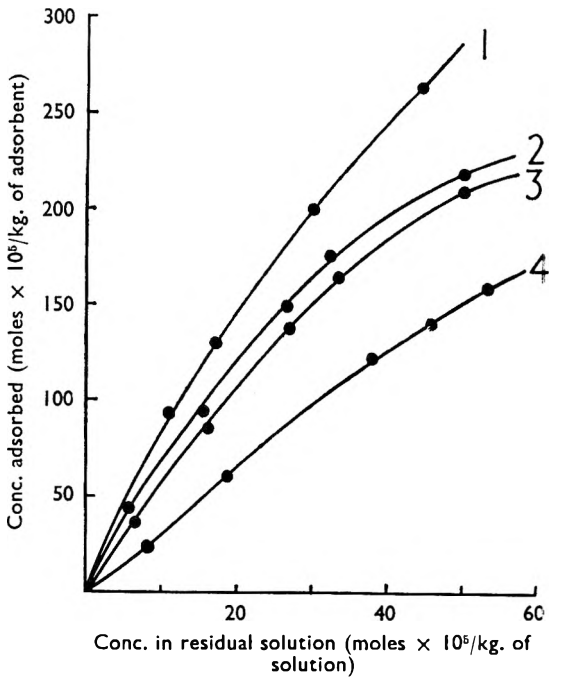


FIG. 3. Adsorption of levorphanol and (+) and (−)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan on levorphanol selective and control adsorbents. (1) Levorphanol, (2) (−)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan, and (3) (+)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan, on levorphanol-selective adsorbent. (4) (+) and (−)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan on control adsorbent.

RESULTS AND DISCUSSION

A silica gel prepared in the presence of levorphanol (II; R = Me) gave, under the prescribed treatment, an adsorbent which adsorbed levorphanol better than its enantiomorph, dextrorphan, and adsorbed both isomers better than did an adsorbent obtained from a gel prepared in the absence of levorphanol (see Fig. 1). Batch to batch variation occurred in the adsorptive power of the adsorbents but the relative adsorption pattern of the isomers was always similar. A gel prepared in the presence of dextrorphan gave an adsorbent which adsorbed dextrorphan better than it did levorphanol (see Fig. 2). The adsorptive power of the adsorbents varied with the time, temperature and humidity

of the storage conditions but the relative adsorption pattern for the isomers on a particular adsorbent did not.

It is considered that the above preparation of stereoselective adsorbents results in "footprints," at the surface of the adsorbent particles, of the reference molecules which were present during the gel formation. The extraction procedure is considered to strip the organic molecules from

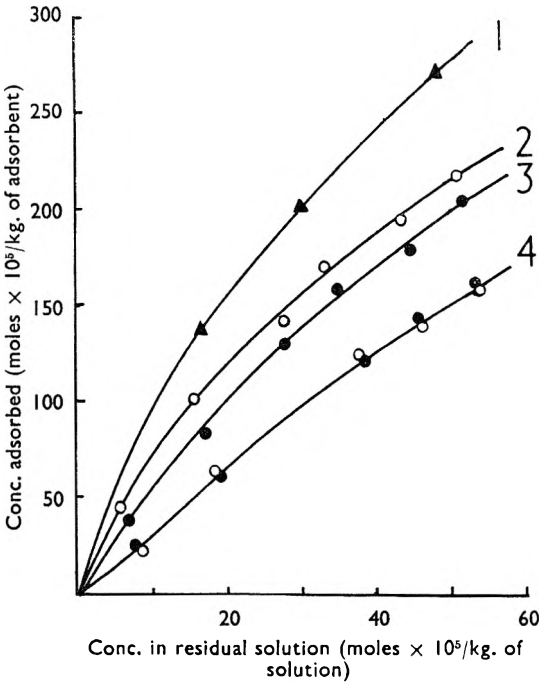


FIG. 4. Adsorption of dextrorphan and (+) and (−)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan on dextrorphan-selective and control adsorbents. (1) Dextrorphan, (2) (+)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan, and (3) (−)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan on dextrorphan-selective adsorbent. (4) (+) and (−)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan on control adsorbent; ○ represents the (+)- and ● the (−)-isomer.

the surface layers to leave imprints and partial imprints with configurational integrity in the hydrated silica surface. A less likely explanation[11] for the selectivity of the adsorbent is that the unextractable (under these conditions) organic molecules left embedded inside the adsorbent set up active points for the association of identical and related molecules. (Detailed discussion of these explanations will be presented elsewhere.)

Irrespective of which explanation be correct, we have already shown that adsorbents which exhibit stereoselectivity may be used for configurational assignments because molecules of like configuration are adsorbed more readily than those of unlike configuration. Consequently an isomer not too dissimilar in structure from levorphanol and of like configuration will be adsorbed better on a levorphanol selective adsorbent

than will its stereoisomer; the opposite adsorption pattern for the two stereoisomers should obtain using a dextrorphan selective adsorbent. In Figure 3 is presented the adsorption of (−)- and (+)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan (III; R = Me) on a levorphanol selective adsorbent; the (−)-isomer is adsorbed more than the (+)-isomer. Figure 4 shows that (+)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan (III; R = Me) is adsorbed more strongly than its enantiomorph

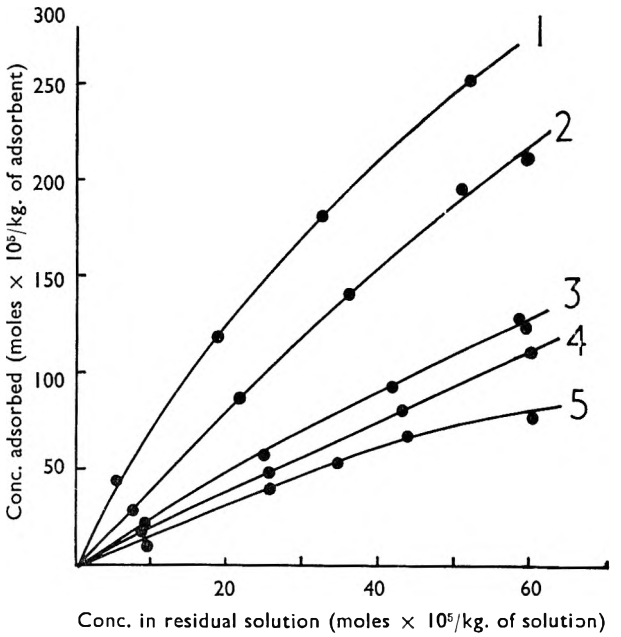


FIG. 5. Adsorption of dextrorphan, levorphanol, and morphine on dextrorphan- and levorphanol-selective adsorbents and on control adsorbent. (1) Dextrorphan on dextrorphan-selective adsorbent and levorphanol on levorphanol-selective adsorbents. (2) Dextrorphan and levorphanol on control adsorbent. (3) Morphine on levorphanol-selective adsorbent. (4) Morphine on dextrorphan-selective adsorbent. (5) Morphine on control adsorbent.

on a dextrorphan selective adsorbent. Similar adsorbents prepared at different times and used after different storage times gave similar relative adsorption patterns for the isomers despite differences in the actual adsorptive power of the adsorbents. It follows that (−)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan (III; R = Me) and levorphanol (II; R = Me) have similar configurations.

Because (−)-phenazocine (III; R = CH_2CH_2Ph) is prepared from (−)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan (III; R = Me) and (+)-phenazocine from the (+)-*N*-methylbenzomorphan by a route not involving the asymmetric centres, identical configurations for levorphanol and (−)-phenazocine are established.

Adsorbents obtained from gels prepared in the presence of (−)-morphine did not exhibit pronounced adsorptive power compared with

that of control adsorbents. Such weak morphine selective adsorbents adsorbed levorphanol slightly more strongly than dextrorphan. The configurational similarity of (−)-morphine (I; R = Me) and levorphanol was thus indicated but further evidence was required. In Figure 5 the uptake of (−)-morphine on levorphanol and dextrorphan selective adsorbents of similar adsorptive power is presented; (−)-morphine is adsorbed more strongly on the levorphanol selective adsorbent than on the dextrorphan selective one. Thus levorphanol and (−)-morphine have similar configurations.

The other (−)-morphinan isomers (II) shown in Table I are known to have configurations identical with that of levorphanol because of their method of preparation from the same precursor isomer. Consequently all the (−)-morphinan isomers shown in Table I have configurations similar to that of (−)-morphine.

The analgesic activity of morphine (I), phenazocine (III; R = CH_2CH_2Ph) and its related *N*-methyl analogue (III; R = CH_3), and racemorphan and its related *N*-alkyl analogues (II), resides substantially in the *laevo* isomers which are shown above to have similar configurations. The importance of the configurational requirements of the analgesic receptor site thus receives further support.

Acknowledgements. The authors thank Dr. E. L. May, N.I.H., Bethesda, U.S.A., for a gift of (−)- and (+)-2′-hydroxy-2,5,9-trimethyl-6,7-benzomorphan. They also thank Roche Products Ltd. for supplies of levorphanol and dextrorphan and Imperial Chemical Industries Ltd. for the sodium silicate solution used in this work.

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After Miss Anderson presented the paper there was a DISCUSSION. The following points were made.

It was possible by use of stereoselective adsorbents to line up the various compounds which had similar configurations. If such arrangements agreed with pharmacological considerations, support would be provided for the analgesic receptor site hypothesis. The authors did not agree with the alternative explanation by Canadian workers. The technique was being used solely to establish configuration. Italian workers had separated isomers by means of stereoselective adsorbents.

PREPARATION OF A ^{35}S LABELLED TRIMEPRAZINE TARTRATE SUSTAINED ACTION PRODUCT FOR ITS EVALUATION IN MAN

By Earl Rosen and Joseph V. Swintosky

From the Smith Kline & French Laboratories, Philadelphia, Pa., U.S.A.

Received May 19, 1960

Multi-pellet sustained action capsules containing radiochemically labelled drug were prepared by a pan coating procedure. Pellets of varying lipid thickness were prepared, and mixed in different proportions to obtain products having variable *in vitro* release characteristics. Capsules were prepared from these pellets for administration to man. Oral doses of 5 mg. of labelled trimeprazine were given in a non-sustained release form at 0, 4, and 8 hours. Other subjects received single 15 mg. oral doses of labelled trimeprazine in sustained action capsules. The ^{35}S activity was determined in blood and urine at selected times. Sustained action capsules were prepared which when evaluated in man gave data for ^{35}S activity in blood and urine which were comparable to that observed for three divided doses of non-sustained release drug administered at 0, 4, and 8 hours.

This study was undertaken to establish a procedure for incorporating ^{35}S trimeprazine tartrate [(±)-10-(3-dimethylamino-2-methylpropyl)-phenothiazine tartrate], an antipruritic drug, into a sustained action capsule; and to acertain, by *in vivo* studies, its *in vitro* release characteristics.

The introduction of sustained action dosage forms of drugs has necessitated the adoption of standards for control of manufactured batches. However, it is apparent that different *in vitro* tests are necessary to evaluate different types of preparations, and that they have no value as an indication of sustained release *in vivo* unless their results can be related to their biological response[1]. Before clinical trials, animal studies may provide qualitative sustained release data; however, ultimate proof rests with human testing. The selection of a suitable objective test depends in part on the methods of analysis that can be applied to the drug. For example, blood concentrations and urinary excretion data are applicable for this purpose with some chemotherapeutic agents[2–4]. Direct measurement of pharmacological or physiological responses has also been useful[5].

For trimeprazine tartrate none of these approaches was found particularly suitable. Therefore, a radiochemical approach was selected, because preliminary studies indicated that it would permit detection and quantitative measurement of ^{35}S in blood serum and urine. In addition the detection of the ^{35}S of trimeprazine tartrate and its metabolities would not be complicated by the host of substances in the urine which frequently complicate other analytical methods.

Since one of the objectives of the work was the direct comparison of sustained release and multiple dose drug administration, satisfactory data could be obtained by measuring only the radioactivity appearing in the blood and urine of subjects receiving these dosage regimens of labelled

drug. This approach assumes that the human body metabolises trimeprazine tartrate in the same manner whether administered in conventional or sustained release form.

Methods and Materials

Source of ^{35}S Trimeprazine Tartrate

(±)-10-(3-Dimethylamino-2-methylpropyl)phenothiazine 9-^{35}S tartrate with a specific activity of 5·6 μc/mg. was made in these laboratories, under the direction of Dr. D. W. Blackburn.

Preparation of Dosage Forms

A disposable glove-box made of plywood and plexiglass and fitted as depicted in Figure 1 was placed in a fume hood with a draft of 1,400 cu. ft./min. The hood was provided with filters to trap particles.

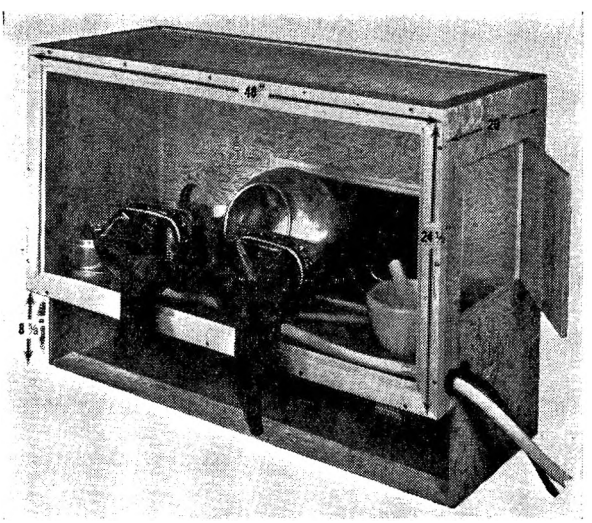

Fig. 1. Picture of glove-box showing position of equipment.

A flexible rubber sheet was inserted in the back wall of the glove-box to provide an air lock entry port for the drive shaft of the motor driving a 12-in. stainless steel coating pan. The glove-box was maintained under a slight positive static pressure of 0·2 in. of water by an air flow of about 2 cu. ft./min. The air was removed through a filter in the back of the box.

Materials Used in Preparation of the Medicated Pellets

31·2 g. of ^{35}S trimeprazine tartrate.
58·8 g. of a 1 : 1 mixture of starch, U.S.P. and powdered sucrose, U.S.P.
900 g. of U.S. No. 16 to 20 mesh sugar pellets.

Water-alcohol gelatin adhesive:

gelatin, U.S.P., 10 per cent w/v.; hydrochloric acid, U.S.P., 0·5 per cent v/v; water, 30 per cent v/v; Alcohol (90 per cent ethanol, 10 per cent methanol) 70 per cent v/v.

Wax-fat Solution:

glyceryl monostearate, N.F., 11 per cent w/w; glyceryl distearate, 16 per cent w/w; white wax, U.S.P., 3 per cent w/w; carbon tetrachloride, U.S.P., 70 per cent w/w.

The 900 g. of sugar pellets were placed in the coating pan. The 31·2 g. of ^{35}S trimeprazine tartrate and 58·8 g. of starch-sugar mixture were placed in the glove-box before sealing the entry port. The water-alcohol gelatin adhesive was placed in the Binks Pressure Vessel under 35 lb./sq. in. gauge pressure. Shoulder length rubber gloves were attached to the glove ports, and the operator transferred the labelled drug to the mortar. The drug was triturated to a fine powder, mixed with the starch-sugar diluent and divided into three approximately equal portions, each in a separate container. Then air was circulated through the box, the coating pan set in motion and the pellets sprayed with the gelatin adhesive. When just wet, after about 2 seconds of spray, using a Binks Flow Gun Model 31V (Binks Manufacturing Co., Chicago, Illinois) fitted with a No. 5 nozzle, the spraying was stopped and the pellets were allowed to roll for about 30 seconds to distribute the adhesive. One of the three portions of powder was then sprinkled on the pellets and the mass mixed by means of a rubber paddle. After 2 minutes of mixing the pellets had taken up all of the powder, the pan was stopped, and the pellets were dried for 5 minutes. The two remaining portions of coating powder were applied in the same way and the pellets were dried for 1 hour, then removed from the pan and screened through a U.S. No. 12 onto a U.S. No. 25 standard mesh sieve to remove lumps and "fines". After screening, the pellets were removed from the glove-box, the motor disconnected from the pan, and the glove-box sealed and removed from the hood. 967 g. of pellets designated Group A were recovered from the screening operation. 750 g. of these were placed in the 12-in. stainless steel coating pan in the fume hood for further processing. The wax-fat solution was placed in the Binks Pressure Vessel fitted with a heating mantle. This solution was maintained under 35 lb./sq. in. gauge pressure at 40°. Using the Binks Flow Gun fitted with a No. 3 nozzle, the wax-fat solution was sprayed at 7 second intervals on the pellets rotating in the pan. Two-minute drying intervals followed each 7-second spraying period. This procedure was repeated 38 times, until the pellets had increased in weight by 105 g. or 14 per cent of the starting weight. This material was designated Group B. 703 g. of Group B pellets were subsequently returned to the pan and a 1·3 per cent increment of wax-fat added. These pellets were designated Group C. A 1·6 per cent increment of wax-fat was then applied to 562 g. of Group C pellets. These pellets were designated Group D. A 2 per cent increment of wax-fat was then

applied to 419 g. of Group D pellets. This material was designated Group E. Each group of pellets was screened through U.S. No. 12 onto U.S. No. 25 standard mesh sieves to remove lumps and fines and sampled by the Sample Splitter described by Souder and Ellenbogen[6]. Representative samples of each group were assayed for ^{35}S trimeprazine spectrophotometrically by ultra-violet absorption over the range 270 to 240 mμ. Samples were then tested for *in vitro* release using USP XV Simulated Gastric and Intestinal Fluids by the method of Souder and Ellenbogen[6].

From these results capsules of the desired release characteristics were made by mixing the various groups. A desired composition was filled by hand into No. 0 hard gelatin capsules. Several capsules were taken at random to be assayed for ^{35}S trimeprazine and *in vitro* release.

Studies on Man

Fourteen adult male inpatients were used, in several of whom one dosage regimen was crossed-over to another. The drug was administered either at 0 hour in the once a day regimen or at 0, 4, and 8 hours in the three divided doses regimen. The dosage regimens were as follows:

5 mg. of non-sustained release ^{35}S trimeprazine, single dose.
15 mg. of non-sustained release ^{35}S trimeprazine, single dose.
5 mg. of non-sustained release ^{35}S trimeprazine, 3 doses in one day.
15 mg. of ^{35}S trimeprazine in sustained release capsules, single dose.

Blood samples were withdrawn at $\frac{1}{2}$, 1, 3, 6, 9, 12, 15, 24 and 48 hours by venipuncture of the anticubital veins. Total urinary output collections were made at 3, 6, 9, 12, 15, 24 and 48 hours. Measurements of radioactivity on the blood serum were by the method of Chen[7]. Serum and urine samples were counted in the Packard Tri-Carb Model 314-DC Liquid Scintillation Spectrometer (Packard Instrument Co., La Grange, Illinois) with the counting chamber set at $-8°$.

Results of the Pan Coating Formulation Technique

Release and assay data for the five bulk pellet groups are given in Table I.

Previous experimentation with laboratory animals, and subjective clinical studies, suggested that a 2:9:9 pellet ratio of groups A, B, and E, respectively, might provide a useful release characteristic in encapsulated pellets. Experimentally, this bulk composition was found to assay 11·3 mg. of ^{35}S trimeprazine per g. (three observations) with release of 18, 37, 70, and 88 per cent by $\frac{1}{2}$, 2, $4\frac{1}{2}$, and 7 hours.

Individual 442 mg. portions of this bulk pellet mixture, equivalent to 5 mg. trimeprazine base, were placed into No. 0 hard gelatin capsules. Each capsule contained approximately 475 pellets. Four capsules from 75 were selected randomly for assay. Average assay results indicated 11·0 mg. trimeprazine per g. (six observations), and a release of 21, 38, 67, and 88 per cent at the respective time intervals.

Capsules of Group A non-lipid coated pellets, each containing 5 mg. of ^{35}S trimeprazine, served as controls for non-sustained release ^{35}S trimeprazine.

Results and Discussion

The preparation of sustained release capsules described in the MacDonnell patent[8] is a coating pan procedure which does not lend itself to the precise laboratory preparation of a single dosage unit. Since the preparation of the medicated pellets before application of the lipid coating is a dusting procedure, it was conducted in a "closed" inexpensive disposable system.

The selection of 900 g. of starting sugar pellets and the concentration of drug in the coating powder were based on the following considerations. Lipid coated groups of less than 400 g. result in unpredictable batch to batch variations of *in vitro* release. Selection of a relatively low dose of ^{35}S trimeprazine (5 mg.) for inclusion in a relatively large capsule results in a low concentration of drug on the pellets and minimises the amount

TABLE I

Release and Assay Data for the Five Bulk Pellet Groups

Pellet groups	Per cent ^{35}S trimeprazine released				Assay* mg./g.	No. of observations
	½ hr.	2 hr.	4½ hr.	7 hr.		
A	100	—	—	—	14·1	8
B	31	59	92	93	12·0	2
C	11	29	86	92	10·2	2
D	10	22	74	90	10·1	2
E	0	12	47	89	9·9	3

* A S.D. of ±0·5 mg./g. was obtained from pooled results of 26 assays.

of radioactivity used in the experiment. Application of coating powder in three or more coats produces a uniform coating of the pellets and good batch to batch reproducibility. The preparation of a minimum of four primary sustained release groups and one non-sustained release group offers the best chance of providing material which can be selected for mixing to give the desired *in vitro* release pattern.

Patient Safety Considerations

Preliminary tests in rats with labelled trimeprazine tartrate showed that over 95 per cent of the ^{35}S administered orally was excreted in the urine and faeces within 96 hours (unpublished). Tests in human subjects receiving unlabelled trimeprazine orally showed that approximately 30 per cent of the administered dose was excreted as phenothiazines, in just the ether extracted portion of the urine, after 24 hours. Therefore, it was considered safe to administer a dose of less that 50 μc to man.

Blood and Urine Studies

The objective studies in man were made by Dr. Philip C. Johnson, Veterans Administration Hospital, Oklahoma City, Oklahoma. Details of the blood, urine, and faecal data after both oral and intravenous administration of ^{35}S trimeprazine tartrate will be published elsewhere.

The following is a summary of the results. The serum levels of radioactivity after administration of four different regimens are plotted in Figure 2 as arithmetic average mg. of trimeprazine. Although we recognise that the radioactivity measured in the blood and urine may emanate from both ^{35}S trimeprazine and its metabolites, we have chosen for convenience to express the values as mg. of trimeprazine to which the ^{35}S is equivalent. Inspection of these curves reveals that after peak serum levels are attained, after single 5 and 15 mg. oral doses of non-sustained release drug, ^{35}S disappearance from the serum follows distinctly similar die-away patterns for both doses. Also it is of interest to note that the areas under these two curves in the 0 to 24 time interval are in the ratio

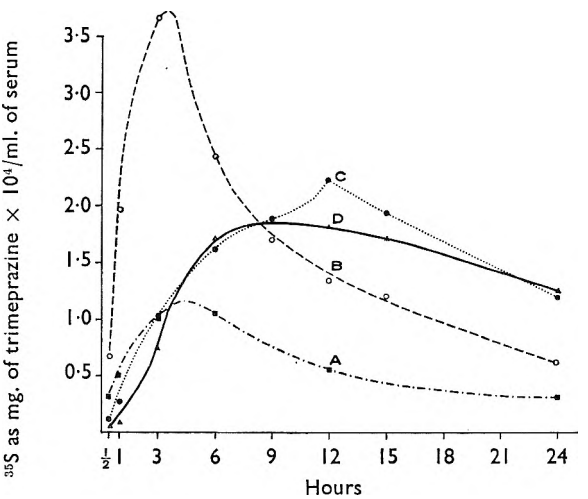

FIG. 2. Average ^{35}S serum levels of adult human subjects after oral administration of labelled trimeprazine: A, 5 mg. once daily to 4 subjects; B, 15 mg. once daily to 4 subjects; C, 5 mg. three times a day every 4 hours (total of 15 mg.) to 5 subjects; and D, 15 mg. sustained release capsules to 5 subjects. The drug was administered at 0 hour in the once a day regimens and at 0, 4 and 8 hours in the three divided doses regimen.

of 1 : 3, indicating a similar percentage absorption from these two regimens. Figure 2 also illustrates the similarity between ^{35}S serum levels observed for a single 15 mg. dose of sustained release drug and three 5 mg. doses of non-sustained release drug given at 0, 4, and 8 hours.

Figure 3 shows arithmetic average cumulative urinary recoveries represented as mg. of the administered doses. Over a 24-hour period the curves for the 5 mg. non-sustained release dose administered at 0, 4, and 8 hours, and the single 15 mg. sustained release dose are nearly straight lines, which indicates a fairly constant rate of ^{35}S urinary elimination over this period. There is no significant difference in the per cent urinary recovery of ^{35}S between the four dosage regimens.

The combined results in Figures 2 and 3 indicate that the ^{35}S serum and urine patterns are very different when one compares the single 15 mg. doses, where one dose is sustained release and the other is not. The 15 mg.

sustained release dose results in a much lower peak serum level, and ^{35}S activity is maintained at a much more uniform level. Also it is apparent that administration of the sustained release product closely parallels the t.i.d. divided dose therapy in ^{35}S serum and urine levels. The composite data of Figures 2 and 3 strongly suggest the therapeutic equivalence of this single 15 mg. sustained release dose administered once in 12 hours, to three 5 mg. non-sustained release doses given at 4 hour intervals. These objective studies have been supported with subjective clinical studies in 460 human subjects receiving unlabelled trimeprazine tartrate sustained release capsules having similar *in vitro* release characteristics.

It is apparent from this study that when a labelled drug can be incorporated into sustained and non-sustained action dosage forms, the development pharmacist has a new useful tool for evaluating performance

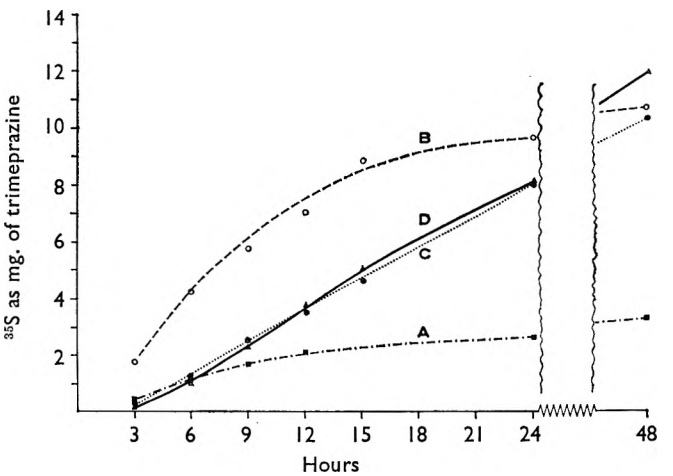

FIG. 3. Cumulative average ^{35}S urinary excretion of adult human subjects after oral administration of labelled trimeprazine: A, 5 mg. once daily to 4 subjects; B, 15 mg. once daily to 4 subjects; C, 5 mg. three times a day every 4 hours (total of 15 mg.) to 5 subjects; and D, 15 mg. sustained release capsules to 5 subjects. The drug was administered at 0 hour in the once a day regimens and at 0, 4 and 8 hours in the three divided doses regimen.

of such products. The biochemical measurements of a drug and its metabolites in human blood and urine are especially valuable in relative comparisons of objective performance. When the development pharmacist has access to such human testing procedures in man, drug product performance can be evaluated quantitatively at the biochemical or pharmacological level, and if these last can be related to therapeutics, establishment of rational *in vitro* release specifications can be accomplished with some confidence.

Acknowledgement. The authors gratefully acknowledge suggestions and technical assistance of a number of colleagues, especially Mr. T. Flanagan, Mr. J. Fitzpatrick, and Dr. S. Free.

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After Dr. Swintosky presented the paper there was a DISCUSSION.

THE ASSIMILATION AND ELIMINATION OF IRON ADMINISTERED ORALLY TO THE DOG AS FERROUS ISOASCORBATE AND FERROUS AMMONIUM SULPHATE

BY H. D. C. RAPSON,* A. A. G. LEWIS† AND JEAN M. COOPS†

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Received May 23, 1960

Radioisotopically labelled iron as ferrous ammonium sulphate or ferrous isoascorbate has been orally administered to dogs. The movement and excretion of the iron has been followed by examination of plasma, faeces and urine samples. There is a maximum in the plasma iron at approximately 1 hour after dosing. Between 2 and 15 per cent of the iron is not recovered in the faeces and no trace is found in the urine. The maximum plasma iron level attained appears, from the limited data obtained, to be linearly related to the amount administered. There is no significant difference between the two forms used. A feature of the work is the use of a simple method to assay the faecal activity, which is described.

THE absorption, distribution and fate of iron after oral administration have been frequently studied by orthodox chemical methods. When compounds are compared in this way, a complete distribution study is always tedious and recoveries are poor. In particular this applies to the recovery of iron from faeces. These difficulties seem to have been minimised by the use of tracer methods.

EXPERIMENTAL

Materials

Ferrous isoascorbate. A preparation of the material was kindly supplied by Mr. E. H. Searle of Product Research Division, Beecham Research Laboratories, together with purified isoascorbic acid, which also was used by the Radio Chemical Centre at Amersham to prepare the labelled material. The final material was freeze dried and sealed in ampoules conveniently containing the equivalent of 20 to 25 mg. of ferrous iron of total activity approximately 15 μc.

Ferrous ammonium sulphate. This material used was analytical reagent grade satisfying the specification in the British Pharmacopoeia 1958. The labelled material was supplied to specification in 10 ml. rubber capped multi-dose bottles by the Radio Chemical Centre at Amersham, 2 ml. containing the equivalent of 20–25 mg. of ferrous iron of total activity approximately 15 μc.

Animals. Two mature and healthy bitches designated M and N were used.

M was a greyhound of 8 years and weight 18 kg. N was a mongrel of 3 years and weight 17·5 kg. Both had been trained to take a stomach tube, and were accustomed to the blood sampling technique. Pens and paws of the animals were monitored regularly for contamination.

Dose and administration of dose. Doses varying from approximately 20 mg. to 150 mg. of ferrous iron were given. The required quantity of material was dispersed in distilled water containing in 100 ml. of water 0·6 ml. of N HCl. The latter was used so that the pH of the dose approximated to that of gastric juice. The dose was given by a stomach tube to the fasting animal. The following precautions were taken to eliminate loss of dose on the walls of the tube and glassware, and to provide a control for assay purposes.

Twice the quantity of labelled iron compound required was dispersed in 200 ml. of the acidified distilled water. The solution was carefully divided into two equal parts. One part was retained as a control. The stomach tube was prewashed by pouring 100 ml. of unlabelled iron compound of concentration similar to that to be administered followed by two 50 ml. portions of distilled water. After allowing approximately 30 seconds for drainage, the tube was inserted in the animal. The solution of labelled compound was then poured down the prewashed stomach tube into the animal, and the vessel washed twice with 50 ml. distilled water, the washings being poured down as well. After allowing approximately 30 seconds for drainage, the tube was withdrawn.

Samples

Faeces and urine. Samples of faeces were collected in wide neck 16 oz. screw topped bottles as soon as they were passed and the time noted. This generally occurred once in every 24 hours. The samples were stored in a refrigerator at 0–5°. Urine samples were taken by catheter in the early part of the work, but these showed so little activity that urine examination was discontinued.

Blood. Samples of 25 ml. to 30 ml. in volume were withdrawn with an hypodermic syringe from leg veins. The blood was centrifuged before clotting to obtain the plasma which was then stored in well stoppered bottles in a refrigerator until assayed.

Activity Assay

The plasma and faeces differed much in activity. This, and their different compositions, made assay by different methods desirable. The usual corrections for background and counter paralysis time were made.

Faeces. A method of assay using the 16 oz. storage bottle was devised to eliminate transfer from the bottles.

The control specimen of labelled iron compound was transferred to a 16 oz. bottle and made up to a constant volume with distilled water. Bottles of faeces were similarly made up and shaken well to disperse the material. The bottles were centrally placed in a counting chamber containing four G10 γ-type counters connected in parallel to a ratemeter. A counting rate reading was taken and the bottle rotated through about 120° when a further reading was made. This process was repeated, three readings in all being taken. These were averaged. The control was assayed before and after the examination of a series of samples and its average activity was used for calculation. This procedure eliminated the

effect of bottle irregularities and gave statistically consistent data on the same sample, when examined in several bottles. Calculations showed that the β-activity of the ^{59}Fe was effectively stopped by the glass.

The counters, if exposed to light, gave spurious results. This effect lasted for over a minute after exposure. The counting chamber was,

TABLE I

SCHEME OF EXPERIMENTAL WORK

Dog	Expt.	Preparation	Dose size as mg. Fe^{++}	Date
M	1	Ferrous ammonium sulphate	22·5	19.8.58
M	2	Ferrous ammonium sulphate	22·5	26.8.58
M	3	Ferrous isoascorbate	22·5	2.9.58
N	4	Ferrous isoascorbate	155·0	15.10.58
N	5	Ferrous isoascorbate	128·0	5.11.58
N	6	Ferrous ammonium sulphate	22·5	10.12.58

therefore, made light-tight and time was allowed for light effects to disappear between the introduction of the sample and the measurement of its activity.

Plasma. The low activity of the samples made it necessary to use a scintillation counter. 10 ml. of plasma was accurately measured into a planchette, which was submitted to the counter. A suitable aliquot of the control sample was diluted so that 10 ml. gave approximately similar activity. The plasma activity was calculated using the dilution factor.

Equipment. A ratemeter was used for the examination of faeces. The scintillation counting equipment consisted of a photomultiplier, an

TABLE II

AMOUNT OF IRON (AS A PERCENTAGE OF THE TOTAL DOSE) NOT RECOVERED IN THE FAECES

Dog	Expt.	Not recovered per cent
M	1	2·56
M	2	2·84
M	3	2·12
N	4	13·60
N	5	10·90
N	6	16·50

E.H.T. unit of high stability, a preamplifier, a linear amplifier with provision for variable discriminator bias voltage and a scalar. Both sets of equipment were standard lines of good quality.

RESULTS

The quantity of radioactive material available for the preliminary experiments was sufficient for six experiments. The scheme of work is shown in Table I.

Excretion of ^{59}Fe in the Faeces

After administration of the dose, faeces were collected regularly; collection and assay was terminated when activity was no longer detected.

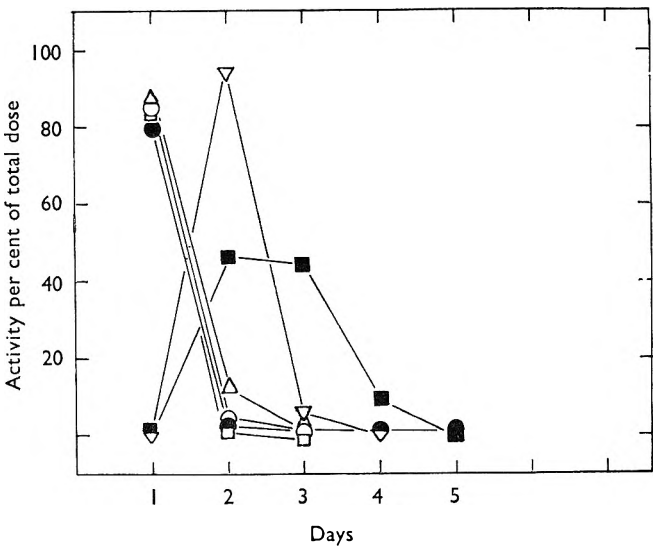

FIG. 1. Daily excretion of iron (as per cent of the total dose) in faeces.

△ Expt. 1. 22·5 mg. Fe^{++} as ferrous ammonium sulphate.
▽ Expt. 2. 22·5 mg. Fe^{++} as ferrous ammonium sulphate.
■ Expt. 3. 22·5 mg. Fe^{++} as ferrous isoascorbate.
● Expt. 4. 155 mg. Fe^{++} as ferrous isoascorbate.
○ Expt. 5. 128 mg. Fe^{++} as ferrous isoascorbate.
□ Expt. 6. 22·5 mg. Fe^{++} as ferrous ammonium sulphate.

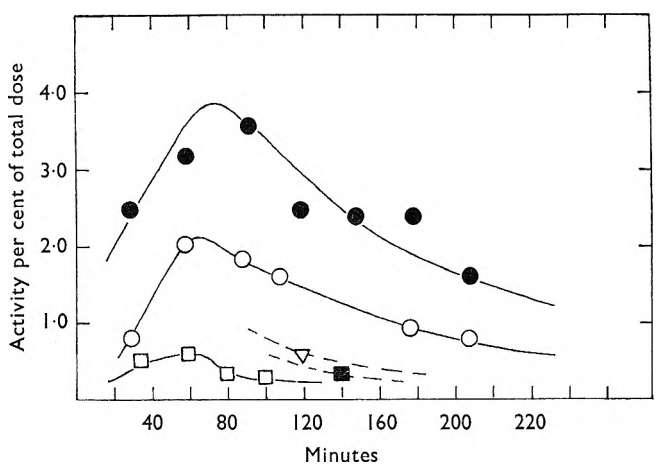

FIG. 2. Variation in plasma iron level (as per cent of the total dose) with time.

▽ Expt. 2. 22·5 mg. Fe^{++} as ferrous ammonium sulphate.
■ Expt. 3. 22·5 mg. Fe^{++} as ferrous isoascorbate.
● Expt. 4. 155 mg. Fe^{++} as ferrous isoascorbate.
○ Expt. 5. 128 mg. Fe^{++} as ferrous isoascorbate.
□ Expt. 6. 22·5 mg. Fe^{++} as ferrous ammonium sulphate.

The samples have been grouped into 24 hour periods to facilitate comparison.

It was originally intended to use one dog only for the work. Dog M, unfortunately, succumbed to an infection and died; the second animal was then introduced. Nevertheless, some of the data obtained on each animal can be compared. In particular experiments 1, 2 and 6 involve similar quantities of iron.

That such comparisons are limited can be seen from Table II, in which the percentage unrecovered is quoted.

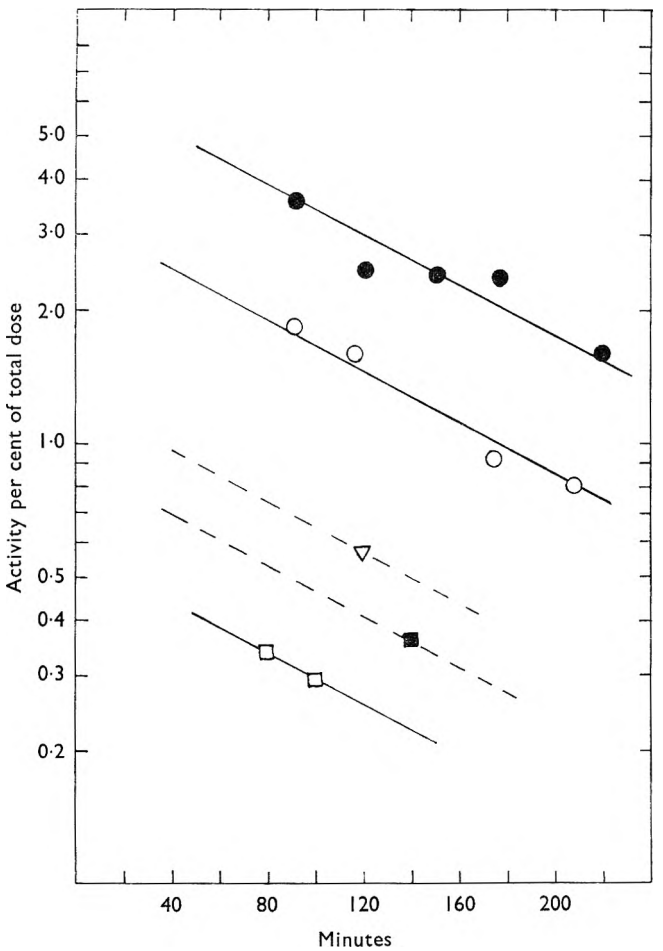

FIG. 3. Decrease in plasma iron level (as a per cent of the total dose) with time from one hour after dosing.

▽ Expt. 2. 22·5 mg. Fe^{++} as ferrous ammonium sulphate.
■ Expt. 3. 22·5 mg. Fe^{++} as ferrous isoascorbate.
● Expt. 4. 155 mg. Fe^{++} as ferrous isoascorbate.
○ Expt. 5. 128 mg. Fe^{++} as ferrous isoascorbate.
□ Expt. 6. 22·5 mg. Fe^{++} as ferrous ammonium sulphate.

Dog N clearly excretes less of the iron administered than dog M. Moreover, from Figure 1 its excretion pattern appears more regular. Here the relative amount of iron excreted is shown as a function of time.

Plasma ^{59}Fe Levels

Initially measurements of plasma activity were made only on samples obtained 2 and 24 hours after administration of the dose. Later as the experimental procedure was developed samples were taken at approximately 30 minute intervals for 4 hours. The results of these measurements are shown in Figure 2. The levels are given as a percentage of the total

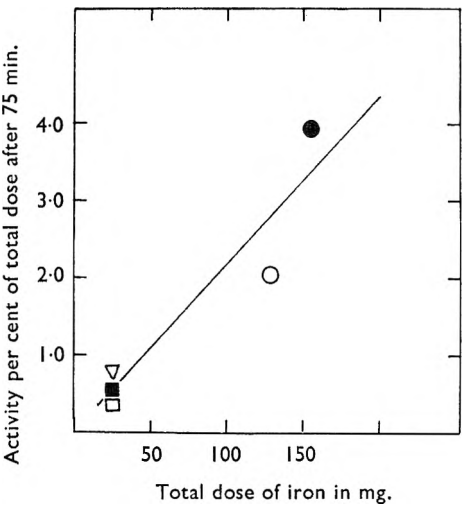

FIG. 4. Variation with dose size of the plasma iron level (as a per cent of the total dose) estimated to exist 75 minutes after dosing.

▽ Expt. 2. 22·5 mg. Fe^{++} as ferrous ammonium sulphate.
■ Expt. 3. 22·5 mg. Fe^{++} as ferrous isoascorbate.
● Expt. 4. 155 mg. Fe^{++} as ferrous isoascorbate.
○ Expt. 5. 128 mg. Fe^{++} as ferrous isoascorbate.
□ Expt. 6. 22·5 mg. Fe^{++} as ferrous ammonium sulphate.

dose administered. This is calculated from the measured activity of the 10 ml. plasma sample by assuming that each animal has a total plasma volume of 800 ml.

Despite the variation in faecal iron recovery the data in Figure 2 appears consistent in that the plasma levels are approximately proportional to the quantity of iron administered. The curves are generally uniform in shape and the few levels obtained on dog M fit in with those from dog N.

The maximum level is reached approximately 75 minutes after administration.

The consistency in plasma level patterns is also supported by the results obtained when the logarithm of the iron level is plotted against time. Figure 3 shows that the relation is linear, and with dog N, where comparison is possible, the lines have similar slopes. Only the plasma levels of samples taken after 75 minutes are given in Figure 4. If the lines are extrapolated to 75 minutes, the plasma level obtained approximates to the

maximum value and this can be used to compare the ease with which a particular preparation is assimilated. The data, so obtained, can be augmented by assuming a similar relationship for the plasma levels obtained earlier in dog M. This assumption is questionable but not unreasonable, particularly when the results of experiments 1, 2 and 6 are compared. Here, identical doses were given and the resulting levels are similar when account is taken of the time of sampling.

The plasma levels at 75 minutes, obtained by this extrapolation procedure, are plotted against the size of the dose in Figure 4.

Qualitatively, bearing in mind the limited amount of data available, there appears to be a linear relationship between the quantity of iron administered and the maximum plasma iron obtained. This relationship does not appear to be influenced by the form in which the iron is presented.

Discussion

The results of some investigations suggest that only a limited section of the intestinal tract in dog[1] facilitates the uptake of iron. Granick[2] states that the most active region is just below the pyloric sphincter. Interpretation of the maximum shown to occur in many plasma level-time curves can of course lead to a similar conclusion. In this work the maximum occurs approximately 1 hour after dosing. Thus, the upper twentieth of the tract appears most active which is consistent with the findings of others.

The animals were fasted for some hours before and after dosing. Consequently the only iron passing through the active region is that from the dose together with a small amount excreted in the bile. However, since the latter is about 1 mg. in 24 hours,[3] for man, it may reasonably be neglected for doses of 22 mg. and over in this present work on dogs.

Thus, after allowing for scatter, the increase in plasma iron appears directly proportional to the dose in the range studied. Whilst it is unwise[5] to accept without question the assumption that the increase in plasma iron is directly related to the iron converted into haemoglobin, many[3,4] consider the increase to be a good measure of this. If this is accepted, then the present results are probably consistent with the contention[3,4,6] that iron uptake is not primarily regulated at any stage in the passage from the lumen to the plasma. The relative amount of iron absorbed does not appear to decrease with increase in the quantity administered. This is interesting, since in the range 1 to 10 mg. iron/kg. weight, a decrease would be anticipated. Here it should be noted that no attempt had been made to simulate iron deficiency in the dogs, though some iron is lost by taking blood samples.

The exact form of the plasma iron level-time curve in the first hour is a matter for conjecture, owing to the limited number of activity measurements in this period. It is clear, however, that continuous monitoring of the blood would permit a more detailed study, based on the form of the curve in the initial period, of the transfer of iron from the lumen to the plasma. This in turn would facilitate a comparison of various iron preparations.

Continuous monitoring of doses as low as 10 μc presents difficulties. Consequently some form of sampling would have to be retained for the measurement of long-term changes in plasma activity, since the latter may fall inside the wider fiducial limits associated with continuous monitoring.

The method employed for faecal activity measurement is, to the best of the authors' knowledge, new. It is considerably less laborious than that previously reported[5,7].

Acknowledgements. The authors gratefully acknowledge the facilities and experimental material kindly supplied by the Royal College of Surgeons, Buckston Browne Farm Experimental Station and the labelled ferrous ammonium sulphate and ferrous isoascorbate supplied by Beecham Research Laboratories Limited.

To this must be added thanks for the assistance given by Dr. D. J. Jenkins of the Radio Chemical Centre, Amersham, who prepared the labelled material for this work.

They are also pleased to acknowledge with thanks the discussions that they have had with their colleagues Dr. E. T. Knudsen and Mr. D. M. Brown.

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After Dr. Rapson presented the paper there was a DISCUSSION.

Short Communication

PHOSPHATIDE MEMBRANES

By L. Saunders

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Received May 23, 1960

An improved understanding of the effects of drugs on cell membranes is likely to follow from the development of realistic models of these membranes. Many attempts have been made but none of the models so far devised have borne much resemblance to the original.

The reaction which produces a cell membrane can be considered as a fundamental process of biochemistry. It seems likely that this reaction is the precipitation of a phosphatide film from the intracellular fluid by calcium and magnesium salts in the outer liquid, and it is significant that the calcium salt content of intra-cellular fluid is extremely small. This initial phosphatide film is then presumably strengthened by deposition of protein and polysaccharide layers. The membrane so formed has a certain mechanical strength but its most important property is its limited permeability to salts and other substances.

The formation of a complete cell membrane is obviously a complex process involving a number of components. It would be a useful advance if this process could be simulated using a limited number of purified substances of known structure and this is a problem which we have been studying for some time. Our early work showed that films of measurable mechanical strength could be formed at the boundary between a phosphatide sol and a salt solution of concentration sufficient to precipitate the sol[1]. Improved chromatographic methods for purifying lecithin showed that the presence of lysolecithin in lecithin preparations had a considerable effect on the properties of the sols[2].

The next stage in the investigation has been an attempt to produce phosphatide films having limited permeability to salts. Once this has been achieved there exists the possibility of obtaining and studying

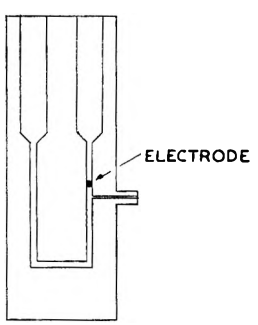

Fig. 1. Diffusion cell.

polarised membranes, a field which should be of interest in the study of nerve impulse and memory storage mechanisms.

Experimental. To study membrane permeability effects the simple perspex apparatus shown in Figure 1, has been used. In this exploratory work, permeability to the precipitating salt only, has been examined. In an extensive series of experiments, boundaries have been formed between a calcium or magnesium chloride solution, usually 10^{-3} N, and a

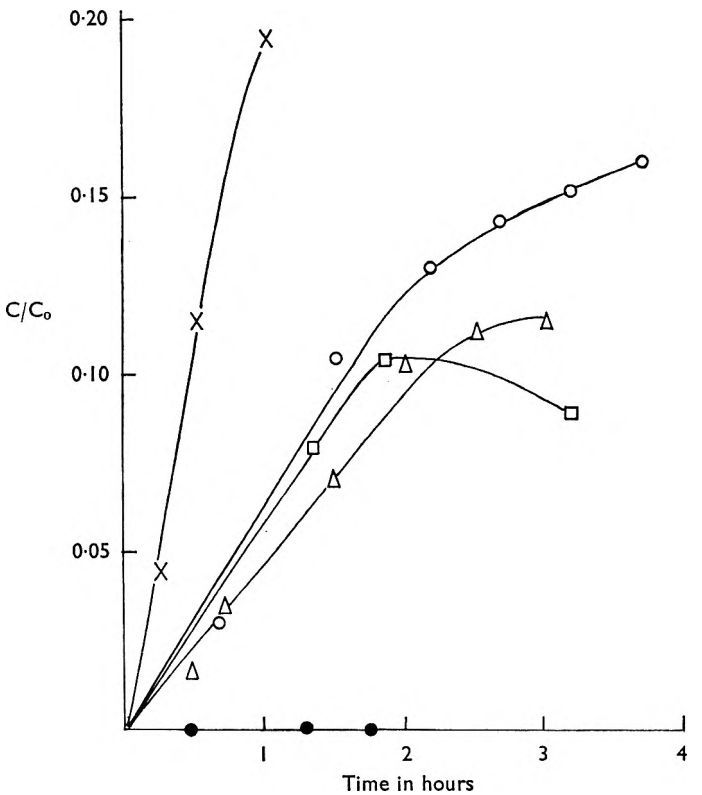

FIG. 2. Diffusion of 10^{-3}N $CaCl_2$. A plot of change in conductivity C, relative to initial conductivity of salt solution, C_0, against the time in hours, at a fixed height above the boundary. X, into water; O, into 2 per cent lec. 0·4 per cent lyso.; △ into 2 per cent lec.; □ into 5 per cent lec.; ● into 10 per cent lec.

phosphatide sol, the sol normally being the liquid below the boundary. Only 1 ml. of sol was required.

Sharp boundaries were formed by causing the sol and solution to flow off together through the exit tube in the left hand arm of the cell. Diffusion of salt into the sol was followed by measuring the conductivity between two platinum electrodes fixed 2 mm. above the boundary. Analar salts and demineralised water were used. The phosphatides were prepared as already described[3,4]. The sols were completely stripped of small electrolytes by treating them with mixed ion exchange resins; this

reduced their conductivity to an extremely low figure which remained unchanged for several days.

Much work has been devoted to attempting to obtain a system in which no diffusion of salt into the sol occurred, that is to prepare a sol impermeable to calcium salts. Since it had been found that 0·5 per cent lecithin sols gave films of measurable strength, much of the earlier work was done with dilute sols of concentration up to 2 per cent. In a number of experiments some retardation of calcium chloride diffusion was noted (see Fig. 2). The influence of additional components such as lysolecithin, cholesterol and plasma albumen was examined but no particularly significant effects were found.

A more concentrated sol containing 5 per cent each of lecithin and serum albumen did show some limited permeability effects. The salt

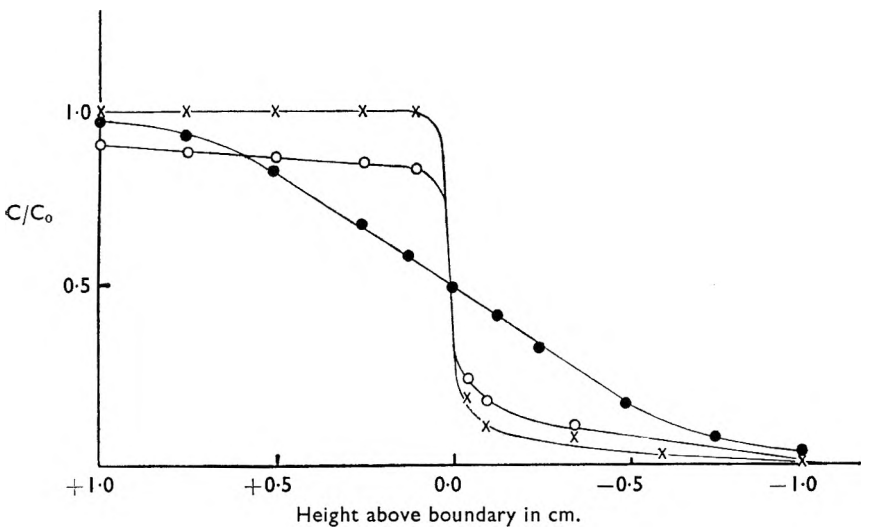

FIG. 3. Plots of conductivity relative to initial conductivity of salt solution C_0 against height above the boundary for 10^{-3} N $CaCl_2$ at 20 hours. ● into water; ○, into 5 per cent sol; X, into 10 per cent sol.

solution conductivity dropped at first, became constant and then began to increase slowly. This indicated that diffusion into the sol had stopped, presumably as a result of the slow formation of an impermeable film at the boundary. A similar effect (see Fig. 2) was obtained with a 5 per cent lecithin sol without albumen.

When these sol-solution systems were left overnight, the boundary remained extremely sharp. Mixed phosphatide sols containing more than 1 part of lysolecithin to 10 of lecithin showed some retardation of diffusion of calcium chloride (Fig. 2) and the boundary remained in its initial position at the exit tube; diffusion of salt into the sol could be seen as an opacity spreading down into the sol. Pure lecithin sols and sols containing a lower proportion of lysolecithin gave an apparent diffusion of salt, since the conductivity readings decreased with time.

However, overnight the boundary fell and it seems that in these systems a relatively impermeable film is formed. Osmotic effects then cause the sol to exude water so that the boundary falls and the salt solution above the boundary is diluted.

An interesting effect was noted with more concentrated lecithin sols (above 5 per cent). On standing alone overnight, a separation into two liquid layers (co-acervation), occurred. The upper layer was clear and the lower layer as a thick liquid containing most of the lecithin.

When the concentration of lecithin in the diffusion cell was increased to 10 per cent, the effect which had been sought throughout this work, was found. No change of conductivity of the salt solution in contact with this sol was observed. To confirm the impermeability of the sol to calcium chloride, a technique for scanning the column in the left hand capillary of the cell, was developed. The solution: sol boundary was formed and left for 20 hours, the liquid in the left hand capillary was then carefully moved up and down the tube and conductivity readings were taken in various positions relative to the sharp boundary. These measurements confirmed the relative impermeability of the 10 per cent sol. Figure 3 shows the results of scanning studies with 5 and 10 per cent sols together with a curve for the diffusion of calcium chloride into water after 20 hours. Immediately below the boundary, the lecithin sol had shrunk away from the sides of the tube slightly leaving a thin layer of clear liquid around it, this is the reason for the measurable conductivity just below the boundary.

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After the Author had presented the paper there was a DISCUSSION.

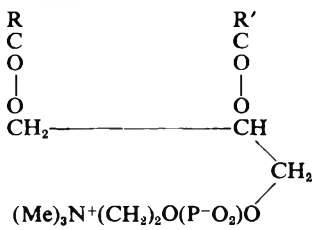
DETECTION OF LYSOLECITHIN IN A SAMPLE OF EGG LECITHIN

BY L. SAUNDERS AND J. PERRIN

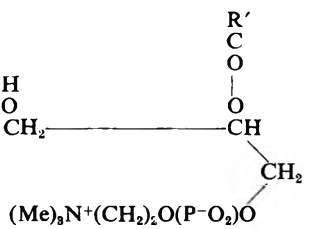
From the Department of Chemistry, School of Pharmacy, University of London, Brunswick Square, W.C.1

Received May 23, 1960

SMALL quantities of lysolecithin alter the physical properties of lecithin sols. This may have disadvantages if the latter is used as a suspending or emulsifying agent; or if it is injected, any lysolecithin impurities may cause unpleasant reactions. It was therefore decided to develop a method for detecting the lyso compound.



Lecithin



Lysolecithin

Methods involving molecular weight determinations are of little use for this purpose because a molecular weight variation of egg lecithin up to 10 per cent may be caused by altering the diet of the hens. Thus, methods such as osmotic pressure determination, and nitrogen and phosphorus percentage determinations are valueless. Dietary variation may also cause substantial variation in the iodine value of lecithin. The difficulty of reacting the primary alcoholic group of lysolecithin with any of the usual reagents is enhanced by possible hydrolysis of the phosphatides during reaction so liberating more primary alcoholic groups, and also because the phosphatides are thermolabile. Attempts to detect lysolecithin in lecithin samples by infra-red studies were also unsuccessful. A successful method has been developed from flocculation studies. These were based on observations made by Thomas[1] and Saunders[2]. Small quantities of lysolecithin protect a lecithin sol from flocculation by electrolytes to varying degrees. By measuring the optical density of a sol flocculated by the addition of electrolytes the presence of lysolecithin can be detected and the amount estimated.

Preparation of lecithin. The ninhydrin reacting impurities were removed by Dowex 1 × 2 50–100 mesh ion exchange resin in a bicarbonate form[3]. The lysolecithin impurities were removed by silicic acid chromatography as in reference 2. The product was recrystallised six times from methylethylketone-acetone (1–3) to give a white solid of 1·0 nitrogen: phosphorous ratio and iodine value 71.

Preparation of lysolecithin. This was prepared by the action of an aqueous solution of Russell viper venom on an ethereal solution of lecithin[2]. After four chloroform-ether precipitations the product was recrystallised twice from warm anhydrous ethanol. The crystallised product had a nitrogen : phosphorous ratio of 1·0 and an iodine value of 2 (approx.).

Infra-red studies. Potassium bromide discs containing 1 per cent lecithin or 1 per cent lysolecithin were prepared and the absorption curves determined by an Infra-cord machine. The curves were very similar and so no simple analytical procedure could be developed.

Coagulation studies. Concentrations of potassium and calcium chloride were chosen similar to those used by Thomas[1]. The optical density of the sols increases as the degree of flocculation increases if measurements are made before the precipitated phosphatides settle. All sols and solutions were prepared using ion exchanged *distilled* water.

TABLE I

TOTAL PHOSPHATIDE CONCENTRATION = 0·1 PER CENT FACTOR FOR KCl SOLUTIONS = 1·035. PERCENTAGE OF LYSOLECITHIN IN THE SOL

KCl conc. × 10^3 M	0	1	2	3	4	5	7·5	10
30·0	0·255	0·225	0·220	0·188	0·170	0·164	0·154	0·152
15·0	0·328	0·281	0·225	0·169	0·152	0·140	0·129	0·128
9·0	0·258	0·238	0·178	0·128	0·122	0·120	0·111	0·109
7·5	0·236	0·219	0·166	0·122	0·188	0·114	0·102	0·103
6·0	0·200	0·184	0·155	0·114	0·102	0·100	0·092	0·088
4·5	0·182	0·158	0·142	0·106	0·098	0·095	0·083	0·081
3·0	0·178	0·118	0·108	0·100	0·082	0·088	0·078	0·072
—	0·105	0·087	0·085	0·086	0·072	0·073	0·070	0·050

Preparation of lecithin sols. The material was dried overnight *in vacuo* and then dissolved in the minimum amount of ether. The requisite amount of water was added to give a sol of twice the strength required in the actual coagulation test. Nitrogen was then bubbled through the mixture (warmed to 30°) for 15 minutes to remove the ether and to give an even dispersion. The sol was then shaken for 30 minutes.

Preparation of lysolecithin sols. The material was dried overnight and then shaken for 30 minutes with the requisite volume of water to give a sol of twice the strength of that required in the test.

Preparation or mixed sols. In each group of tests the total phosphatide concentration was kept constant and sols of known lysolecithin content were prepared by mixing the correct ratio of lecithin and lysolecithin sols of this same concentration.

For example, if a sol containing 90 per cent lecithin and 10 per cent lysolecithin is required with a total phosphatide content of 0·4 per cent then 90 ml. of a 0·4 per cent lecithin sol is mixed with 10 ml. of a 0·4 per cent lysolecithin sol. In the tests the prepared mixed sols were diluted with an equal quantity of electrolyte solutions and thoroughly mixed. The optical densities developed after known time intervals were measured.

In the preliminary investigations 30 → 3·0 × 10^{-5} M $CaCl_2$ and 30 → 3·0 × 10^{-3} M KCl concentrations and the total phosphatide concentration was varied to give a suitable optical density range. KCl was found to be more suitable than $CaCl_2$ and Thomas[1] also found that lysolecithin was more effective in protecting lecithin sols against the flocculating action of potassium chloride than against the flocculating action of calcium chloride. 0·25 and 0·125 per cent phosphatide sols gave optical densities (up to 1·2) which tended to vary with time. 0·1 per cent sols were found to have suitable optical densities which reached a constant value in 2 hours (up to 0·3).

Measurements of optical density. Table I was compiled by measuring the optical densities of 0·1 per cent phosphatide sols 2 hours after preparation by means of a Spekker absorptiometer. The monochromatic source was a mercury vapour lamp together with a Hilger 605 filter (yellow-green). Five ml. samples were used in 0·5 cm. cells. All experiments were carried out at room temperature (20° ± 1·0°).

As can be seen from the Table up to 4·0 per cent lysolecithin appears to give incomplete protection to the lecithin against the electrolyte concentrations used and so the optical density tends to vary according to the protection afforded. No further protection appears to be afforded by further additions of lysolecithin and thus the optical density remains almost constant. 15 → 7·5 × 10^{-3} M KCl concentrations give the largest changes of optical density, with small lysolecithin additions and so they are more suitable for the detection of small lysolecithin impurities in a lecithin sample.

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3. Saunders and Perrin, *ibid.*, 1960, **12**, 253.

After Mr. Perrin presented the paper there was a DISCUSSION.

THE SIZE, SHAPE AND HYDRATION OF CETOMACROGOL 1000 MICELLES

By P. H. Elworthy

From The School of Pharmacy, Royal College of Science and Technology, Glasgow

Received May 19, 1960

Cetomacrogol solutions have been studied by three techniques. The micellar weight in water has been found from light-scattering experiments to be 101,000; the micelles appear to be spherical. Viscosity measurements can be interpreted in terms of micellar hydration, and 1·96 g. water appear to hydrate 1 g. of cetomacrogol. A similar amount of hydration (1·92 g. water per g. cetomacrogol) can be calculated from the diffusion coefficient. A check on the micellar weight was obtained from the diffusion and viscosity results, giving 96,000, in good agreement with the result from light-scattering.

Non-ionic surface-active agents generally consist of a hydrocarbon chain to which is linked a series of water attracting groups. Cetomacrogol 1000 has the structure:

$$Me[CH_2]_{15 \text{ or } 17}\,[OCH_2CH_2]_{20-24}OH$$

and the chain of ethylene oxide units forms the hydrophilic portion of the molecule.

Few determinations have been made on the micelle size of non-ionic detergents. Triton X100, which has the structure

$$(Me)_3CCH_2C(Me)_2C_6H_4[OCH_2CH_2]_{10}OH$$

has been found by Kushner and Hubbard[1] to have a micellar weight of 90,000, determined by light-scattering; this corresponded to 140 monomers in the micelle. The micelles appeared to be spherical.

As cetomacrogol has a much longer chain of ethylene oxide units than Triton X100, a greater interaction with the solvent would be expected than that given by the latter substance. In this paper results from viscosity, diffusion, and light-scattering experiments have been used to gain an idea of the size, shape, and hydration of cetomacrogol micelles.

Experimental

Materials

A commercial sample of cetomacrogol, based on cetyl alcohol, was dried before use. The molecular weight, determined by freezing point depression in benzene, was 1210.

Organic solvents were Analar materials, and were dried and fractionated. Toluene gave $n_D^{25} = 1{\cdot}4940$ and benzene $n_D^{25} = 1{\cdot}4972$; Timmermans[2] gives 1·4941 and 1·4981 respectively.

The sodium dodecyl sulphate was a pure sample kindly given by Dr. I. L. Thomas. (Percentage S found = 11·10, percentage S calculated = 11·12.)

Diffusion Coefficients

Diffusion measurements were made using the Gouy diffusiometer. Results for cetomacrogol have been reported[3], but their interpretation has not been discussed.

Viscosity Measurements

Viscosities of solutions relative to water were determined in a suspended level dilution viscometer.

Light Scattering

An apparatus was constructed based on that described by Parreira and Ottewill[4], and by Robinson[5].

A modification to the thermostated cell holder has been made (Fig. 1). This consisted of a circular brass tank, with a copper coil sealed in its base. Water from an external thermostat was circulated through the coil.

A block of perspex was used as a secondary light scattering standard.

Clarification of solutions. Solutions were filtered through No. 5 on three sintered glass discs under pressure. The first few ml. were discarded.

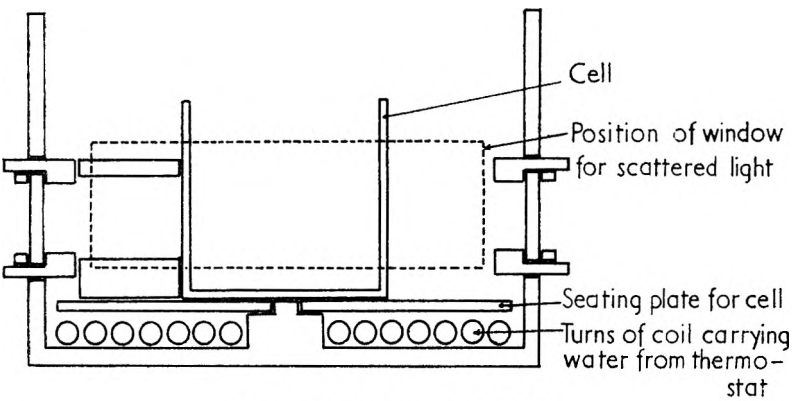

FIG. 1. Thermostated cell holder.

With detergent solutions five or six filtrations were necessary to free the solutions from dust.

Specific refractive index increment. The refractive index difference between solution and solvent was determined using a Hilger-Rayleigh interferometer, and the technique described by Bauer for monochromatic light[6]. The specific refractive index increment could be calculated from the refractive index difference.

Calibration of apparatus. After obtaining regular scattering envelopes for dilute Ludox solutions (a colloidal silica preparation), the calibration constant of the apparatus was determined using Ludox solutions sufficiently dilute to avoid the corrections of Maron and Lou[7]. The calibration constant, C, was determined from the optical density, D, and the amount of light scattered at 90° to the incident beam, S_{90}, by the same solution. S_{90} was measured with reference to the perspex block.

$$T = \frac{16\pi}{3}.R_{90} = \frac{2{\cdot}303D}{1} = C.S_{90}$$

where T is the turbidity of the solution, R_{90} is Rayleigh's ratio, 1 is the length of the cell of solution. C had the value $4{\cdot}76 \times 10^{-4}$ cm^{-1}.

Density of Solid Cetomacrogol

Determinations by a displacement technique in a dry light petroleum fraction gave a mean of 1·143 g./ml.

Results

The diffusion coefficient of cetomacrogol in water has been found[3] to be only slightly concentration dependent, and the diffusion was virtually free from charge effects. The extrapolated value of the diffusion coefficient at zero solute concentration was $5{\cdot}10 \times 10^{-7}$ cm.^{2}sec.$^{-1}$.

TABLE I

Relative Viscosities of Cetomacrogol Solutions at 25°

Concentration, per cent	0·254	0·313	0·418	0·668	0·928	1·005
η_r	1·018	1·023	1·031	1·050	1·076	1·080
Concentration, per cent	1·256	1·552	1·550	1·669	1·856	2·320
η_r	1·105	1·132	1·135	1·143	1·164	1·209
Concentration, per cent	2·500	2·899	3·868	—	—	—
η_r	1·233	1·280	1·400	—	—	—

The viscosities of cetomacrogol solutions relative to water are given in Table I.

Increasing the cetomacrogol concentration has a fairly large effect on the viscosity of the solutions.

Before studying the light-scattering of cetomacrogol solutions, the apparatus was checked by measuring R_{90} for benzene and toluene. Values of $16{\cdot}2 \times 10^{-6}$ and $17{\cdot}7 \times 10^{-6}$ cm.$^{-1}$ respectively were obtained against $16{\cdot}4 \times 10^{-6}$ and $17{\cdot}6 \times 10^{-6}$ cm.$^{-1}$ found by Ottewill and Parreira[4]. Depolarisations of 0·42 and 0·43 respectively were found.

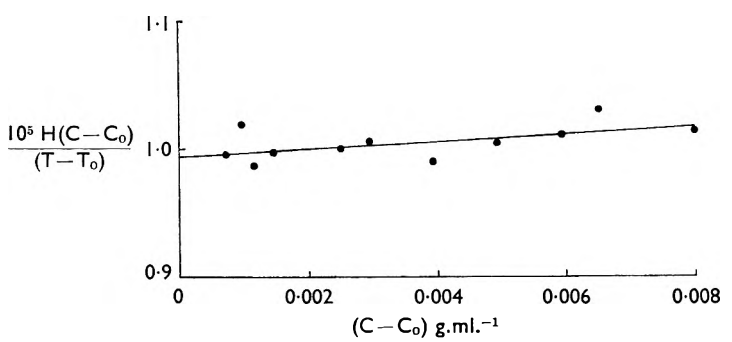

Fig. 2. Graph of $H(C-C_0)/(T-T_0)$ against $(C-C_0)$. For symbols see text.

As a further check the micellar weight of sodium dodecyl sulphate in 0·04N sodium chloride solution was also determined, giving 28,600 at 25°, compared with Phillips and Mysels' value[8] of 29,600 at 22°. The specific refractive index increment, dn/dc for the compound, was found to be 0·116 ml. g.$^{-1}$, compared with 0·115 ml. g.$^{-1}$ by Phillips and Mysels.

Cetomacrogol in water gave dn/dc = 0·133 ml. g.$^{-1}$ (±0·001 ml. g.$^{-1}$).

The results of the light-scattering experiments are shown in Figure 2; corrections for depolarisation were applied to the results. A plot of

concentration against turbidity in the low concentration region is shown in Figure 3. At very low concentrations, the turbidity of the solution is scarcely more than that of the solvent, but a point is reached where turbidity begins to increase rapidly with increasing concentration. This is due to micelle formation; as turbidity is proportional to molecular weight of the solute, micelles scatter much more light than single molecules. A crude estimate of the critical micelle concentration, from a large-scale drawing of Figure 3 (including three additional pre-CMC points not

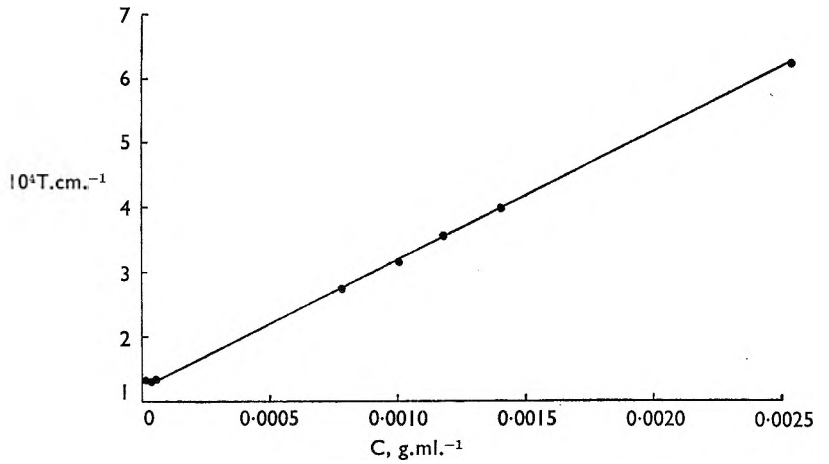

FIG. 3. Graph of turbidity of solutions of cetomacrogol, T, against concentration, C.

shown in the figure) is 0·006 per cent. From surface tension, solubilisation, and the iodine method, it was found[9] that the critical micelle concentration lay in the region 0·006 to 0·008 per cent, in agreement with the value from light scattering.

DISCUSSION

For solutions of small, approximately spherical molecules, the molecular weight, M, is related to the turbidity by

$$\frac{HC}{T} = \frac{1}{M} + 2BC$$

where B is the second virial coefficient, C is the concentration, and

$$H = \frac{32\pi^3 n_0^2 (dn/dc)^2}{3N\lambda^4}$$

where λ is the wavelength of the light used, N is Avogadro's number, and n_0 is the refractive index of the solvent. On plotting HC/T against C, a straight line should be obtained with an intercept of 1/M and a slope of 2B.

Following Debye[10,11], for detergents whose solutions give a turbidity T_0 at the critical micelle concentration C_0:

$\frac{H(C-C_0)}{(T-T_0)}$ is plotted against $(C-C_0)$ (Fig. 2).

For cetomacrogol the plot is linear, and the molecular weight, determined from the intercept, is 101,000 ± 5 per cent. As the monomer has a molecular weight of 1210, the micelle contained 83 monomers. From the intensity of scattered light measured at two equal angles about 90° to the incident beam, e.g., 60° and 120°, an idea of the micellar shape can be obtained. Spherical micelles should give equal intensities at the two angles, and the ratio of the intensities (the dissymmetry, Z = intensity at 60°/intensity at 120°) should be unity. Dissymmetries varying between 1·02 and 1·05 were observed experimentally, indicating that the micelles are reasonably spherical. Dust in the solutions and the presence of stray light in the apparatus may lead to results suggesting high dissymmetries. The difficulty of completely clarifying detergent solutions, which tend to suspend particles, is probably responsible for the small apparent residual dissymmetry. Soap micelles under normal conditions are generally considered to be spherical; for spherical micelles of dodecyl pyridinium chloride[4], dodecyl pyridinium bromide[4], and sodium dodecyl sulphate[8,12], dissymmetries of the order of 1·01 to 1·3 have been obtained. Stray light or dust are probably responsible where high Z values have been obtained.

The second virial coefficient, B, has the value $1{\cdot}3 \times 10^{-4}$. Ionised detergents give large values of B, e.g., for sodium dodecyl sulphate B is 122×10^{-4} in water and $4{\cdot}2 \times 10^{-4}$ in 0·1N sodium chloride solution. Cetomacrogol, being unionised gives a lower value for B, namely $1{\cdot}3 \times 10^{-4}$.

The interpretation of diffusion and viscosity results is generally complicated by the effects of shape and solvation on the kinetic behaviour of the particle in solution. Assymmetric or solvated particles diffuse slower than spherical or unsolvated particles of the same molecular weight. Similarily, solutions of assymmetric or solvated particles have higher viscosities than those of spherical or unsolvated ones.

The Einstein equation[13] relates the specific viscosity, η_{sp} to the volume fraction of the solute, ϕ.

$$\eta r - 1 = \eta_{SP} = 2{\cdot}5\phi$$

A plot of η_{sp}/ϕ against ϕ should give a straight line with an intercept of 2·5. An intercept greater than 2·5 suggests that the particle is either solvated or assymmetric. In the present study a value of $8{\cdot}1_2$ was found (Fig. 4). As the light-scattering results show the micelle to be spherical, the deviation of $(\eta_{sp}/\phi)_{\phi=o}$ from 2·5 can be considered to be due to hydration only. The extent of hydration can be found from equations of the type given by Oncley[14]:

$$\left(\frac{\eta_{SP}}{\phi}\right)_{\phi=0} = 2{\cdot}5\left(1 + \frac{w}{\bar{v}\rho}\right)$$

where v = specific volume of the solute, ρ = density of solvent, and w is the number of g. of water hydrating 1 g. of cetomacrogol. In this case w = 1·96 g. water, which is a large amount of hydration compared with materials like the proteins, where 0·3 g. water per g. protein is a normal figure.

A further idea of the degree of hydration may be gained by using the diffusion coefficient to calculate the radius of the micelle, r. For spherical particles the Stokes-Einstein equation may be applied:

$$r = \frac{RT}{6\pi\eta ND}$$

η is the viscosity of the solvent, D is the diffusion coefficient, and the remaining symbols have their usual significance. The diffusion coefficient of $5{\cdot}10 \times 10^{-7}$ cm.^{2}sec.$^{-1}$ gives a particle radius of 48·1 Å; the volume of the micelle will thus be 466,000 Å^3. This volume will be occupied by both water and cetomacrogol molecules. The 83 monomers of cetomacrogol will occupy a volume of 146,000 Å^3 (83 × monomer volume calculated from molecular weight and density) leaving a volume

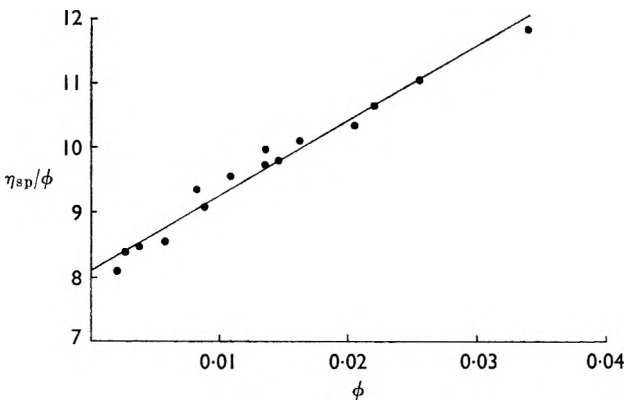

FIG. 4. Graph of η_{sp}/ϕ against ϕ. For symbols see text.

of 320,000 Å^3 for the water. This would allow 10,700 water molecules to be present in the micelle, which corresponds to 129 water molecules per monomer of cetomacrogol, or 1·92 g. water per g. cetomacrogol, in good agreement with the viscosity result. Triton X100 micelles were found to contain 50 water molecules per monomer[1]; but the micelles of this detergent are smaller than those of cetomacrogol. The hydration of Triton X100 corresponds to five water molecules per ethylene oxide unit, while that for cetomacrogol corresponds to six for each —OCH_2CH_2—. This is too large a number to be clustered round each ether linkage, and perhaps the best way of looking at the hydration is to consider that the water molecules are mainly jammed in the interstices of the hydrophilic chains.

A check on the micellar weight can be obtained by combining the diffusion and viscosity results. The hydration of the micelle will make its frictional coefficient, f, larger than that of an unhydrated micelle (f_0) of the same molecular weight. The ratio of the frictional coefficients can be calculated from:

$$\frac{f}{f_0} = \left(1 + \frac{w}{\rho}\right)^{\frac{1}{3}}$$

giving $f/f_0 = 1{\cdot}48_1$.

The diffusion coefficient of the unhydrated micelle (D_o) is obtained from $D/D_o = f_o/f$
giving $D_o = 7{\cdot}55_3 \times 10^{-7}$ cm.^{2}sec.$^{-1}$.
Substitution in that form of the Stokes-Einstein equation for molecular weight yields a result of 96,000 $\pm$ 5 per cent, or a micelle containing 79 monomers, in good agreement with the result from light scattering.

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After the Author presented the paper there was a DISCUSSION.

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