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Vol. X. Supplement **December, 1958**

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BRITISH PHARMACEUTICAL CONFERENCE

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BRITISH PHARMACEUTICAL CONFERENCE

NINETY-FIFTH ANNUAL MEETING, LLANDUDNO, 1958

REPORT OF PROCEEDINGS

OFFICERS:

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H. G. ROLFE, B.Sc., F.P.S., F.R.I.C., London.
E. F. HERSANT, B.Pharm., Ph.D., F.P.S., F.R.I.C., London.

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. ADAMSON, F.R.I.C., Greenford.
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W. H. LINNELL, D.Sc., Ph.D., F.P.S., F.R.I.C., London.
*G. H. HUGHES, M.P.S., Colwyn Bay.
J. B. LLOYD, M.P.S., Manchester.
*H. STEINMAN, M.P.S., Manchester.

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

PROCEEDINGS OF CONFERENCE

LLANDUDNO, 1958

THE OPENING SESSION

The opening session of the Conference was held in the Pier Pavilion in Llandudno, on Monday, September 15, with Mr. D. W. Hudson, President of the Conference (President of the Pharmaceutical Society) in the Chair. On the platform were the Chairman of the Conference (Dr. G. E. Foster), the Chairman of Llandudno Urban District Council (Councillor H. Neville, Chairman of the Local Committee), the Vice-Chairman and Secretary of the Local Committee (Mr. E. Fearnhead and Mr. M. H. Thomas), the Honorary General Secretaries together with members of the Conference Executive Committee.

The President introduced the Chairman of Llandudno U.D. Council, who welcomed the Conference to Llandudno. The President thanked Councillor Neville on behalf of the Conference for his welcome.

The President then handed over the further conduct of the Conference to the Chairman (Dr. G. E. Foster), who delivered his address entitled "Modern Analytical Chemistry in the Service of Pharmacy and Medicine," which is printed in full in the *Journal of Pharmacy and Pharmacology*, 1958, **10**, Supplement, pages *9 T–23 T.*

On the proposition of Mr. S. G. E. Stevens, seconded by Mr. H. G. Moss, the Conference accorded a hearty vote of thanks to the Chairman for his address.

CIVIC RECEPTION

On the evening of Monday, September 15, a Civic Reception was given at the Winter Gardens. The guests were received by the Chairman of Llandudno U.D. Council and Mrs. Neville. A dance was held after the reception.

THE SCIENCE SESSIONS

Meetings were held on Monday, Tuesday, Wednesday and Friday, September 15, 16, 17, 19 at the Town Hall or the Imperial or Grand Hotels, the Chairman presiding. During the sessions the following 28 papers were communicated:—

1. The Basis for "Sufficient of a Suitable Bacteriostatic" in Injections. By G. Sykes, M.Sc., F.R.I.C.
2. The Factors Influencing Sterilisation by Low Pressure Steam. Part I. Design and Instrumentation. Part II. The Influence of Water Content of Cotton Gowns on Equilibrium Times. By T. E. Barson, F. Peacock, E. L. Robins, B.Pharm., A.R.I.C. and G. R. Wilkinson, F.P.S.
3. The Colorimetric Determination of Morphine in Galenical Preparations. By C. A. Johnson, B.Pharm., B.Sc., F.P.S., A.R.I.C. and Cecilia J. Lloyd, B.Sc.
4. Some Observations Concerning the Chemical Reactions Occurring Between Formaldehyde and Peptone. By. K. Bullock, M.Sc., Ph.D., F.P.S., F.R.I.C. and V. Subba Rao, D.Sc. (Andhra), Ph.D. (Manchester), A.R.I.C.
5. The Effects of Added Peptone on the Bactericidal Action of Solutions of Formaldehyde. By K. Bullock, M.Sc., Ph.D., F.P.S., F.R.I.C. and V. Subba Rao, D.Sc. (Andhra), Ph.D. (Manchester), A.R.I.C.
6. The Stability of Solutions of 5-Hydroxytryptophan. By J. W. Hadgraft, F.P.S., F.R.I.C., Shirley A. P. Price, B.Pharm., M.P.S. and G. B. West, B.Pharm., D.Sc., Ph.D., F.P.S.
7. Studies on 5-Hydroxytryptamine and 5-Hydroxytryptophan. By G. B. West, B.Pharm., D.Sc., Ph.D., F.P.S.
8. The Precisions of Some Procedures in Pharmaceutical Analysis. Part I. The Use of a Pipette and a Burette. By A. R. Rogers, B.Pharm., B.Sc., F.P.S., A.R.I.C.
9. Neuromuscular Blocking Agents. Part II. The Preparation and Properties of a Series of *NSN*- and *NNN*-Trisethonium Compounds. By D. Edwards, B.Sc., F.P.S., A.R.I.C., J. J. Lewis, M.Sc., F.P.S., J. B. Stenlake, B.Sc., Ph.D., F.P.S., A.R.I.C. and M. S. Zoha, M.B., B.S.
10. Neuromuscular Blocking Agents. Part III. Some Linear *NNNN*-Tetraethonium Compounds. By D. Edwards, B.Sc., F.P.S., A.R.I.C., J. J. Lewis, M.Sc., F.P.S., J. B. Stenlake, B.Sc., Ph.D., F.P.S., A.R.I.C. and M. S. Zoha, M.B., B.S.

11. Some Aspects of the Property of Angle of Repose of Powders. 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.
12. The Flow Properties of Powders under Humid Conditions. By D. J. Craik, B.Sc. and B. F. Miller.
13. Observations on Increased Disintegration Times of Tablets on Storage. By R. A. Ramsay, F.P.S.
14. The Preparation and the Antibacterial and Antifungal Properties of Some Substituted Benzyl Alcohols. By D. V. Carter, B.Sc., P. T. Charlton, B.Sc., Ph.D., F.R.I.C., A. H. Fenton, B.Pharm., M.P.S., A.R.I.C., J. R. Housley, B.Sc., Ph.D. and B. Lessel, B.Pharm., Ph.D.
15. The Interaction of Chelating Agents with Bacteria. Part I. 8-Hydroxyquinoline (Oxine) and *Staphylococcus aureus*. By A. H. Beckett, B.Sc., Ph.D., F.P.S., F.R.I.C., A. H. Vohora, B.Sc., Ph.D. and Ann E. Robinson, B.Pharm., Ph.D.
16. The Determination of Phenolic Compounds in Pharmaceutical Preparations Using 4-Aminophenazone. By C. A. Johnson, B.Pharm., B.Sc., F.P.S., A.R.I.C. and R. A. Savidge, A.R.I.C.
17. Viscosity Studies with Phosphatide Sols. By I. L. Thomas, B.Pharm. and L. Saunders, D.Sc., Ph.D., F.R.I.C.
18. Vegetable Purgatives Containing Anthracene Derivatives. Part X. A New Active Glycoside of Senna. By J. W. Fairbairn, B.Sc., Ph.D., F.P.S., F.R.I.C., F.L.S., C. A. Friedmann, M.Sc., D.Phil., F.R.I.C. and H. A. Ryan, B.Sc., F.R.I.C.
19. Anatomical Studies in the Genus Digitalis. Part I. The Anatomy of the Inflorescence of *D. purpurea L.* By P. S. Cowley, B.Pharm., M.P.S. and J. M. Rowson, M.Sc., Ph.D., F.P.S., F.L.S.
20. Factors Influencing Percutaneous Absorption. By D. H. O. Gemmell, B.L., F.P.S. and J. C. Morrison, B.Sc.
21. The Actions of Digitalis Leaf Preparations and of Cardiac Glycosides on the Isolated Right Ventricle of the Guinea Pig. By G. A. Stewart, B.Sc., A.R.I.C.
22. Vegetable Purgatives Containing Anthracene Derivatives. Part IX. An Aloin-like Substance in *Rhamnus purshiana* D.C. By J. W. Fairbairn, B.Sc., Ph.D., F.P.S., F.R.I.C., F.L.S. and V. K. Mital, B.Pharm., Ph.D. (Zurich).
23. The Isolation and Comparison of Pyrogenic Factors from *Proteus vulgaris*. By J. P. Todd, Ph.D., F.P.S., F.R.I.C., J. A. M. Shaw, B.Sc. (Pharm.), M.Sc., M.P.S., A.R.I.C., J. A. Blain, B.Sc., Ph.D., A.R.I.C. and W. Boyle, B.Sc.
24. The Surface Activities of α- and β-(Acyl) Lysolecithins. By N. Robinson, M.Sc., A.R.I.C. and L. Saunders, D.Sc., Ph.D., F.R.I.C.
25. A Note on the Pharmacology of Rescinnamine and Serpentine. By M. S. Zoha, M.B., B.S., S. M. Kirpekar, M.Sc.Tech. (Bombay) and J. J. Lewis, M.Sc., F.P.S.
26. A Comparative Study of the Hydrolytic and Non-hydrolytic Methods for the Assay of Solanaceous Drugs. By R. E. A. Drey, B.Sc., A.R.I.C.
27. Identification of Seeds from Various Species of *Strophanthus*. By W. G. Thomas, M.Sc., F.P.S. and C. Melville, B.Pharm., Ph.D., F.P.S.
28. The Functional Groupings of Cucurbitacin E (α-Elaterin). By J. N. T. Gilbert, B.Pharm., F.P.S. and D. W. Mathieson, B.Sc., Ph.D.

The papers are printed in full with reports of discussions in the *Journal of Pharmacy and Pharmacology*, 1958, **10**, Supplement, pages *40 T–256 T.*

THE SYMPOSIUM SESSION

A symposium on "The Evaluation of New Drugs" was held on Thursday, September 18. The CHAIRMAN presided. The introductory paper was presented by Drs. L. G. Goodwin and F. L. Rose. The meeting is reported in the *Journal of Pharmacy and Pharmacology*, 1958, **10**, Supplement, pages *24 T–39 T.*

PROFESSIONAL SESSIONS

With the President of the Conference, Mr. D. W. Hudson, in the chair, professional sessions were held on the mornings of Tuesday, September 16, when Dr. J. C.

Parkinson and Mr. G. T. Espley read introductory papers to the subject "The Pharmacist's Responsibilities to his Postgraduate Student," and Friday, September 19, when Messrs. A. R. G. Chamings and G. T. M. David read introductory papers to the subject "The Presentation of Dispensed Medicines." Full reports of the papers and discussions were published in *The Pharmaceutical Journal*, 1958, **181**, 206–210, 221–224, 254–258, 273–274.

THE CLOSING SESSION

The closing session of the Conference was held on Friday, September 19, in the Pier Pavilion, Llandudno, the Chairman presiding.

Vote of Thanks to the Local Committee

The Chairman called on Mr. H. P. Corrigan to propose a vote of thanks to the Local Committee. This was seconded by Mr. J. J. Lewis. The Chairman then presented to the Angelsey, North Caernarvonshire and Colwyn Bay Branch an enscribed gavel provided from the Bell and Hills Fund. Mr. H. Neville (Chairman of the Local Committee) replied to the vote of thanks and acknowledged the gift.

Annual Report

Mr. H. G. Rolfe presented the following Annual Report of the Executive Committee.

Your Executive have pleasure in presenting the ninety-fifth 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 74 members elected by the Executive who are not members of these Societies.

Conference Research Papers.—The Executive decided this year, as an experiment, to invite authors to submit either the usual full paper or a short communication. Thirty-four papers were submitted; nineteen full papers and nine short communications were accepted for presentation to the Conference. The Executive thank the authors for their contributions.

Bristol Meeting, 1957.—The report of the meeting of the Conference at Bristol was published in the nineth 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 125.

Journal of Pharmacy and Pharmacology.—The Executive has been represented on the Editorial Board by the Chairman (Dr. G. E. Foster), Professor K. Bullock and the Senior Honorary General Secretary.

Future Meetings.—An invitation will be presented at this meeting for the Conference to meet in Bournemouth during the week commencing September 21, 1959.

Several Branches of the Society have made preliminary enquiries regarding the possibility of entertaining the Conference in future years and the Executive are grateful for all these offers of hospitality.

Officers and Executive of the Conference.—Your Executive has nominated the following Officers for 1958–1959:—

Chairman: H. Treves Brown, *Vice-Chairmen:* R. R. Bennett, H. Deane, H. Humphreys Jones, T. E. Wallis, H. Brindle, B. A. Bull, Norman Evers, A. D. Powell, H. Berry, H. B. Mackie, G. R. Boyes, H. Davis, J. P. Todd, K. Bullock, F. Hartley and G. E. Foster. *Honorary Treasurer:* H. G. Rolfe. *Honorary General Secretaries:* E. F. Hersant and D. Train.

Other members of the Executive:—

As more than six persons were nominated for membership of the Executive, an election was held during this meeting of the Conference. The result is that the following will be the elected members of the Executive for 1958–1959—D. C. M. Adamson, A. W. Bull, J. G. Dare, W. H. Linnell, J. B. Lloyd, J. B. Stenlake.

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 1958–1959.

Mr. W. J. TRISTRAM proposed the acceptance of the report and the election of officers of the Conference for the ensuing year. Dr. H. S. Bean seconded.

Mr. H. TREVES BROWN thanked the Conference on behalf of the newly-elected officers.

TREASURER'S REPORT

Mr. H. TREVES BROWN presented and proposed the adoption of the following Report and Statement of Accounts for the year 1958:—

In presenting the accounts for the year ended December 31, 1957, there is one item of special importance to which I must call attention, namely the question of income tax on our investment income.

It has been assumed in the past that the Conference was not liable to income tax but unfortunately this assumption was incorrect and agreement was reached with the Inspector of Taxes that the arrears of tax due up to the end of the year 1956 amounted to £69 15*s.* 6*d.*

The Income and Expenditure Account now shows all interest gross and, as an item of expenditure, the income tax for the current year. For 1957 the total tax payable amounted to £24 6*s.* 6*d.*, and of this sum £17 2*s.* was deducted at source, leaving a balance due to the Inland Revenue of £7 4*s.* 6*d.* Since neither this sum nor the arrears, together amounting to £77, had been paid before the end of the year, they are shown in the Balance Sheet as a liability. The Balance Sheet also shows the deduction from the Accumulated Fund as at December 31, 1956, as it appeared in last year's Balance Sheet, to take account of the tax liability. It also includes the Local Committee Fund, consisting of a donation of £250 from the London Committee 1953 held by the Executive to assist a Branch if necessary in the initial stages of preparation for a Conference. A loan of £250 was made to the Llandudno Local Committee in November, 1957, in respect of the 1958 Conference. The subscriptions from elected members including a composition fee from the Pharmaceutical Society of Northern Ireland amounted to £138 15*s.* and were credited to the account of the *Journal of Pharmacy and Pharmacology*.

H. TREVES BROWN,
Honorary Treasurer.

BRITISH PHARMACEUTICAL CONFERENCE ACCOUNT

INCOME AND EXPENDITURE ACCOUNT, 1957

Expenditure	£	*s.*	*d.*	*Income*	£	*s.*	*d.*
Minute Book—memento to Bristol and District Branch	12	17	0	Interest on 2½% Consols	40	5	0
Replica of Chairman's Badge and engraving	7	7	0	Interest on 3% Savings Bonds	6	0	0
Income Tax for the year	24	6	6	Interest on P.O. Savings Bank Account	11	1	4
Surplus carried to Balance Sheet	62	15	10	Donation from Pharmaceutical Society of Northern Ireland	25	0	0
				Donation from Pharmaceutical Society of Ireland	25	0	0
	£107	6	4		£107	6	4

BALANCE SHEET AT DECEMBER 31, 1957

Liabilities	£	*s.*	*d.*	*Assets*	£	*s.*	*d.*
Accumulated Fund, as at 31.12.56	1,926	15	10	Investments at cost (*a*) £1,610 2½% Consols (Donation by the late Alderman Clayton of Birmingham) (Market value at 31st December, 1957: £749)	1,250	0	0
Less: Arrears of Income Tax	69	15	6				
	1,857	0	4				
Add: Surplus 1957	62	15	10				
	1,919	16	2				
Income Tax Due	77	0	0	(*b*) £200 3% Savings Bonds 1960–70 (Market value at 31st December, 1957: £151)	200	0	0
Local Committee Fund:—							
Donation from London Committee, 1953	250	0	0	Stock of Replicas (3) of Chairman's Badge	20	12	6
				Loan to Llandudno Local Commitee	250	0	0
				Post Office Savings Bank Account	426	14	10
				Cash at Westminster Bank	63	8	10
	£2,246	16	2		£2,246	16	2

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

July 28, 1958

The President seconded, and the Report was adopted.

REPORT OF PROCEEDINGS

BRITISH PHARMACEUTICAL CONFERENCE

INAUGURAL MEETING HELD AT NEWCASTLE-ON-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-ON-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-ON-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, Dès. 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*
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1931	MANCHESTER ..	J. H. FRANKLIN.	R. G. EDWARDS.
1932	ABERDEEN ..	H. SKINNER.	H. M. DUGAN.
1933	LONDON ..	C. H. HAMPSHIRE, M.B., B.S., B.Sc., F.R.I.C.	H. N. LINSTEAD.
1934	LEEDS	C. H. HAMPSHIRE, 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. RUSHTON.
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.	———
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1950	GLASGOW ..	A. D. POWELL, F.R.I.C.	A. OFFICER.
1951	HARROGATE ..	H. BERRY, B.Sc., Dip. Bact. (London), F.R.I.C.	R. W. JACKSON.
1952	NOTTINGHAM ..	H. B. MACKIE, B.Pharm.	W. E. NEWBOLD. Miss G. M. WATSON.
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.
1955	ABERDEEN ..	J. P. TODD, PH.D., F.R.I.C.	G. L. DICKIE.
1956	DUBLIN ..	K. BULLOCK, M.Sc., PH.D., F.R.I.C.	D. J. KENNELLY.
1957	BRISTOL ..	F. HARTLEY, B.Sc., PH.D., F.R.I.C.	E. GEORGE.
1958	LLANDUDNO ..	G. E. FOSTER, B.Sc., PH.D., F.R.I.C.	M. H. THOMAS.

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Closing Session (continued).

PLACE OF MEETING FOR 1959

Mr. H. RIDEHALGH on behalf of Bournemouth and District Branch, extended an invitation to hold the Conference in Bournemouth in 1959.

Mr. H. NOBLE proposed that the invitation be accepted, and Miss J. ALLEN seconded. The vote was put to the meeting and unanimously carried.

VOTE OF THANKS TO CHAIRMAN

Mr. C. W. RIDOUT proposed a vote of thanks to the Chairman.

Mr. W. TALVAN REES seconded. The vote was put to the meeting by the President and carried with acclamation.

Dr. FOSTER 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.

G. E. FOSTER

Chairman, 1958

BRITISH PHARMACEUTICAL CONFERENCE LLANDUDNO, 1958

Chairman: G. E. FOSTER

CHAIRMAN'S ADDRESS

MODERN ANALYTICAL CHEMISTRY IN THE SERVICE OF PHARMACY AND MEDICINE

As with chemistry itself it is impossible to trace the origins of chemical analysis which must have slowly developed from ancient times, through the Middle Ages, and, as a result of the efforts of the founders of scientific chemistry, to have burst into full development during the nineteenth century. One of the first recorded analytical reagents, however, must surely be Pliny's[1] reagent for testing for iron in verdigris by using a papyrus soaked in extract of gall nuts. Although modified by numerous workers[2] Pliny's test has survived for 2,000 years and is still used in vinegar works for the detection of iron in vinegar. In literature and art there are many references to early chemical operations and the practice of iatro-chemistry (the study of chemical phenomena in order to obtain results of medicinal value) during the sixteenth century led workers in medicine and pharmacy to be closely associated with chemical investigations. Indeed, it has sometimes been possible to gain valuable knowledge on chemical procedures from works of art, illustrating early pharmaceutical laboratories. It is therefore not surprising that pharmacists have always been associated with analytical chemistry and particularly with the examination of drugs and their preparations. The names of some early pharmacists have found a place in chemical literature. Scheele (1742–1786), the Swedish pharmacist most famous for his discovery of chlorine, was also author of the method of obtaining hydrogen sulphide from iron sulphide and acid, a reaction which every schoolboy must associate with chemical analysis. The French pharmacist Baumé (1728–1804) has his name associated with the hydrometer and Mohr (1806–1879) is famous for his invention of the burette.

During the nineteenth century it was realised that many vegetable drugs contained active principles and much effort was expended in attempts at their isolation. Pharmacists played a prominent part in this work and achieved notable successes, of which the greatest were probably the isolation of morphine by Sertürner[3] in 1805 and of quinine by Pelletier and Caventou[4] in 1820. It was a natural development that estimation of the active principles in vegetable drugs should be undertaken and gradually pharmaceutical analysis emerged as a section of analytical chemistry. From these early beginnings has sprung the analytical control of pharmaceutical products, largely covered to-day by official and semi-official books of standards, such as the British Pharmacopoeia and the British Pharmaceutical Codex. Much may be learnt of the advances in drug analysis during the past fifty years by an examination of the various

editions of these publications. The B.P. 1898 consisted of monographs dealing with descriptions of official drugs and methods for making their preparations, little attempt being made to assay the products or even to test them for impurities. Some advance was made in the B.P. 1914, but it was in the B.P. 1932 that definite steps were taken to test official preparations for impurities, to standardise them by the inclusion of assay processes and to adopt limits for the content of active ingredients. This trend has continued until to-day the Pharmacopoeia uses appropriate methods covering a wide field of biology, chemistry and physics for the standardisation of its preparations.

While the establishment of classical pharmaceutical analysis was due to the achievements of nineteenth century workers, the present time at the middle of the twentieth century, is appropriate for reviewing the work of later analysts and assessing their contributions to pharmacy and medicine. A close study of modern analytical techniques shows that their great contribution to chemistry has been their increased sensitivity resulting in a reduction in the amount of material required for analysis. This goal has been reached by the development of a number of distinct analytical procedures and it is some of these, having great influence on pharmaceutical progress, that I wish to discuss in the present address.

The Rise of Microchemistry

Like many other activities the development of microchemistry has experienced a series of sporadic and disconnected advances due to the efforts of forceful personalities who brought their ideas before the scientific world. In the case of microchemistry this has given rise to some confusion as to what the term means. Nearly all the early recorded attempts to devise microchemical methods were associated with the microscope, which was used to examine small samples, as an aid to their identification, or for the observation of chemical reactions between drops of solutions on a microscope slide. Thus microchemistry was often regarded as that part of analytical chemistry concerned with the study of chemical reactions with the aid of a microscope. This field, however, is what we would to-day describe as chemical microscopy.

It was the invention of the microbalance early in the present century which set the scene for the great growth of what is now universally known as microchemical analysis. The names of Emich[5], Kuhlmann and Pregl[6] will for ever be associated with this advance. It was when Pregl saw a microbalance constructed by Kuhlmann in Emich's laboratory that he realised that slight modification would provide him with a balance suitable for organic microanalysis. Soon Kuhlmann had made a balance capable of taking a load of 20 g. and weighing 1 microgram. This enabled Pregl to develop his system of microchemical analysis, employed particularly for the determination of carbon, hydrogen and nitrogen in organic compounds. His system was an immediate success and became used in laboratories all over the world.

There have been many modifications in technique since Pregl did his fundamental work and these have been described in terms which have caused no little confusion regarding the size of the sample used. The Committee on Nomenclature[7], Analytical Division of the American Chemical Society, has recommended that the prefixes *macro-*, *semimicro-*, *micro-* and *ultramicro-* should be differentiated according to the size of the sample. *Macro-* methods use samples of 100 mg. or more, *semi-micro-* methods use samples of less than 100 mg. and more than 10 mg., *micro-* methods use samples of from 10 mg. to 0·1 mg. and *ultramicro-* methods use samples of less than 0·1 mg. These recommendations have been widely accepted.

Some Applications of Microchemical Analysis

Progress in medicine and the allied profession of pharmacy is intimately associated with advances in organic chemistry, which has undergone such tremendous growth within living memory. From 1900 until the end of the first world war organic chemists added much to our knowledge of natural products such as alkaloids, sugars and proteins; the chemical constitutions of many of these were established and some were synthesised. Substantial quantities of these materials could readily be isolated and subjected to analysis by *macro*-methods. In the period between the two world wars a new note was struck and the organic chemist became increasingly interested in natural products of a different type. The hormones and vitamins became major topics of investigation and in every case these substances occurred in minute amounts in nature and much effort was needed in order to isolate even a few milligrams before chemical research on composition and structure could commence. Many of those who are familiar with the B.P. 1953 and are used to handling grams or kilograms of aneurine hydrochloride, ascorbic acid, riboflavine, progesterone, testosterone, oestrone and related products may not realise that 30 years ago these were available only in small quantities or had not yet been isolated. Those were stimulating days and I can well remember the hundredweights of yeast and rice polishings worked up for vitamin B_1, the crates of oranges and lemons for vitamin C and the gallons of urine, which arrived in milk churns from maternity homes, for extraction of sex-hormones. Some idea of the yields obtained can be judged from the fact that about 5 grams of vitamin B_1 was isolated from a ton of rice polishings. How then was it possible for the compositions and structures of these products to be determined and their syntheses achieved? It was the development of microchemical analysis which enabled such work to be carried out with the extremely small amounts of materials available and, as a result, these compounds, obtained by total or partial synthesis are now found in hospitals, surgeries and pharmacies throughout the world. Microchemistry is unlikely to make a more useful contribution to pharmaceutical chemistry than it did during those inter-war years.

Microchemistry consists of ordinary chemistry carried out on a small scale and its great value depends upon this factor. Some drugs are very expensive, particularly when first introduced into medicine, and

analytical control of such products may on this account be very costly. Microchemical analysis enables the drug needed for analysis to be reduced to about 1/20th of that required by *macro*-methods and a substantial reduction in the cost of analysis is thereby achieved. The advantages of micro-methods were quickly appreciated by the British Pharmacopoeia Commission, for in the monograph on Ergotoxine Ethanesulphonate, included in the B.P. 1932, it is stated under one test requiring 1 gram of drug that methods of microanalysis, if of equivalent accuracy, may be substituted for this determination. Similar concessions have now become generally accepted in other books of standards. Although not strictly analysis, the use of a microbalance to weigh International Standard Preparations for use in biological assays effects a considerable saving of valuable materials.

The scope of microchemistry in pharmacy is emphasised by Table I, in which are summarised the human doses of a number of potent drugs.

TABLE I

Drug	Human dose
Adrenaline	0·1 to 0·5 mg.
Methadone hydrochloride	5 to 10 mg.
Aneurine hydrochloride	2 to 5 mg.
Atropine sulphate	0·25 to 1 mg.
Carbachol	0·25 to 0·5 mg.
Vitamin B_{12}	50 to 100 μg.
Digoxin	0·25 to 0·5 mg.
Ergometrine maleate	0·5 to 1 mg.
Menaphthone	1 to 5 mg.
Picrotoxin	0·6 to 6 mg.
Stilboestrol	0·5 to 2 mg.
Physostigmine salicylate	0·6 to 1·2 mg.
Glyceryl trinitrate	0·5 to 1 mg.

Generally one dose will be the amount of drug contained in one tablet or a single dose ampoule of injection and it is immediately obvious that a large number of products will be required if analysis is to be attempted by *macro*-methods. In cases where only single tablets or ampoules are available microchemical techniques afford the means of satisfactory analysis. Analysts in the control laboratories of pharmaceutical houses are frequently faced with problems of this nature arising from customers' complaints, the activities of drug addicts and incidents due to overdosage of drugs. Early in the second world war two tablets, found on a German prisoner, were referred to us in the hope that we might be able to identify them. By microchemical analysis, and some good fortune, we were able to state that the tablets consisted of Amphetamine Sulphate, 10 mg. A rather interesting application was the careful removal of a deposit, which had developed on the surface of some tablets returned to us, and its identification by microanalysis as cocoa butter, a constituent of the tablet base.

Without perhaps realising it, workers engaged on pharmaceutical analysis make considerable use of microchemical methods. The well known limit tests for lead and arsenic are obvious examples, while the use of organic reagents for the detection and estimation of trace metals is not uncommon in the B.P. and B.P.C. Diphenylthiocarbazone is used

in the separation of lead in many limit tests for this element, for the detection of mercury in dihydroxyanthraquinone (B. Vet. C.) and for the estimation of zinc in insulin preparations. Traces of chromium in menaphthone are detected by the use of diphenylcarbazide; methyltrihydroxyfluorone is employed for testing titanium dioxide for contamination with antimony while the use of thioglycollic acid for the identification and estimation of traces of iron is well known. In all cases the amounts of metals detected are well within the *ultramicro-* range; in some the sensitivity is very great and of the order of 1 part in 5 million.

In the alkaloidal field volumetric and gravimetric *macro*-methods are used largely for the assay of vegetable drugs and only in special circumstances are colorimetric or spectrophotometric methods, capable of estimating milligrams or less of active ingredients, employed. For the

TABLE II

Alkaloid	Reagent	Approximate weight needed for estimation
Morphine	Radulescu[58] (formation of nitroso derivative)	0·2 to 0·5 mg.
Atropine	Vitali[57] (treatment with nitric acid followed by alcoholic potash)	0·025 to 0·15 mg.
Strychnine	Malaquin[58] (reduction and treatment with sodium nitrite)	0·02 to 0·1 mg.
Ergot alkaloids.. ..	M. I. Smith[59] (*p*-dimethylamino-benzaldehyde)	0·05 to 0·1 mg.
Tubocurarine	Folin-Ciocalteu[8] (lithium and sodium molybdophosphotungstate)	0·05 to 0·1 mg.

assay of alkaloidal preparations containing small doses, however, microchemical techniques depending on specific colour reactions are often used. Table II includes a selection of well-known alkaloids, the reagents employed for their colorimetric estimation and the weight of each needed for a determination. Little difficulty is experienced in obtaining accurate results by these methods when tablets and injections containing pure alkaloidal salts are examined, but they are equally applicable to crude products if the alkaloids are first separated in a form suitable for analysis. The potentialities of these micro-techniques were well illustrated when a single ampoule containing 10 mg. of tubocurarine chloride in 1 ml. was used[8] to determine the strength of the solution by measurement of its optical rotation and by colorimetric assay, after which sufficient injection remained for a biological assay by the rat phrenic nerve-diaphragm method. A further example is afforded by the work of Silber and Bischoff[9], who carried out a very extensive investigation of the alkaloidal content of single sclerotia from crops of ergot, cultivated under varying conditions.

The accurate filling of ampoules is important and Scott[10] has described an apparatus for the precise measurement of the volume of an injection in a single dose container, usually holding 1 ml. of solution. The basis of the method is the direct measurement of the volume by use of a calibrated capillary tube, the liquid from 10 determinations being collected

in a burette for the purpose of assessing the average volume per ampoule. As a final example, reference may be made to the problem of filling insulin zinc suspensions large volumes of which have to be maintained in a state of uniform suspension during filling operations. In this case the variation in the suspension filled may be checked by taking samples of filled vials during the entire filling operation of the batch and carrying out nitrogen determinations by the micro-Kjeldahl method.

Microbiological Assay

For many years pharmacologists have standardised biologically drugs for which no established chemical or physical assays existed and, for this purpose, experimental animals such as rabbits, guinea pigs and especially rats and mice have been used. In more recent times, however, it has been found that, like animals, many micro-organisms need certain nutritional requirements for their growth and by the choice of suitable micro-organisms these requirements may be exploited for analytical purposes. These new analytical tools have indeed earned the description of "micro" both on account of their physical size and for the *ultramicro*-quantities of antibiotics, vitamins and amino acids which may be estimated by their aid. Microbiological methods possess many advantages; no expensive animal houses are required, the costs of animals and their feeding stuffs are eliminated and quite simple apparatus such as is found in a bacteriological laboratory is all that is needed. The time factor is an important consideration. For example, the assay of a vitamin using rats may take six weeks; the same assay may sometimes be carried out microbiologically in as many days and it is understandable that, where possible, microbiological assays have replaced the earlier animal techniques. The procedures of importance in pharmaceutical analysis fall into two categories, concerned with antibiotics and essential growth factors respectively, according to whether the substance examined inhibits or stimulates the growth of the test micro-organisms. It is convenient to refer to them under separate headings.

Antibiotics

The clinical importance of antibiotics depends upon their ability to combat the growth of bacteria and assay procedures are designed to measure this antibacterial effect. The potency of an antibiotic is usually estimated by comparison with that of a standard preparation to which an arbitrary potency in units is allotted. Standard preparations of important antibiotics are available from the Department of Biological Standards, National Institute for Medical Research, London. The essential requirements for an assay are a strain of bacteria highly sensitive to the antibiotic under test together with a culture medium in which the micro-organisms readily grow. Portions of the culture medium inoculated with bacteria under standard conditions are treated with varying, but accurately measured, amounts of antibiotic and incubated; the extent of bacterial growth in the resulting cultures may be used to assess the potency of the antibiotic. This seemingly simple procedure

has proved difficult to control in practice and many modifications have been described. In fact, no one modification is satisfactory for all purposes but three have become established and are widely used. These are (i) dilution methods, (ii) turbidimetric methods, (iii) diffusion methods.

Dilution methods are based upon the standard bacteriological procedure for examination of the effect of antiseptics upon micro-organisms. Falling dilutions of the antibiotic to be assayed in suitable nutrient medium are set up in tubes or plates and inoculated with equal amounts of the test organism. Controls containing no antibiotic are included and these together with the dilutions are incubated and inspected at intervals for bacterial growth. The minimum concentration of antibiotic which inhibits growth is recorded and by carrying out simultaneous tests upon the standard preparation and the unknown the potency of the latter is estimated. A full account of the assay of penicillin by the serial dilution technique has been given by Pope and Stevens[11].

The turbidimetric method is similar in design but the inhibition of bacterial growth is not estimated by an "all or none" end point but by a graded diminution in growth which extends over several tubes, in which it is usual to use liquid nutrient medium. Turbidities, due to bacterial growth in the medium, are measured and a curve is drawn relating turbidity to concentration of antibiotic. A calibration curve, constructed from readings obtained similarly using the standard preparation, is employed for assessing the potency of the sample tested. Bond and Davies[12] have given a critical account of this method of assay.

A solid nutrient medium inoculated with the test organism either in bulk or on the surface is used in diffusion methods of assay. A solution of the antibiotic is applied to a small area of the medium, which is incubated at once or after remaining at a low temperature for some hours. Growth of the organisms in the vicinity of the area of application is inhibited and the distance which the inhibition extends from the latter is related to the concentration of the antibiotic. The seeded medium may be held in test tubes, measured volumes of the antibiotic solution being placed on the surface and allowed to act by vertical diffusion. The assays are, however, more frequently carried out by horizontal diffusion, in which case the antibiotic solution is applied to a seeded plate and a circular zone of inhibition obtained. The height of the column of inhibition or the diameter of the circular zone is directly related to the logarithm of the antibiotic concentration. The latter method was developed extensively by the Oxford workers under Florey's leadership and they have published a very full account of the technique[13]. For purposes of assay the solution under test may be placed in cups cut in the agar medium with a cork borer or it may be filled into small open glass or porcelain cylinders standing upright on the agar. Some workers use filter paper discs or simply apply measured drops to the surface of the medium. It must be remembered that the diameter of the zone of inhibition is related to the concentration of the antibiotic in the case of the cup and the cylinder procedures; with the paper disc or the drop method it is the actual amount of antibiotic which is the determining factor.

These assay methods have been applied to many antibiotics; Table III gives a selection and also indicates the test organisms used.

Essential Growth Factors

Williams[14], in 1919, suggested that the growth of yeast might be used for the assay of "vitamine", but it was not until recent years that the nutritional requirements of micro-organisms were used for the assay of vitamins, especially those of the B group. For this advance we are indebted particularly to Barton-Wright[15] and to Snell[16], who have given detailed descriptions of assay procedures.

The principle of the assay is similar to that used for antibiotics excepting that stimulation instead of inhibition of growth is measured. A suitable culture medium, containing all essential growth factors excepting that to be assayed, is inoculated with the test organism. Aliquots of the seeded

TABLE III

Antiobiotic	Method of assay	Test organism*
Bacitracin	Cylinder plate	*Micrococcus flavus* (N.C.T.C. 7743)
	Turbidimetric	*Staphylococcus aureus* (A.T.C.C. 10537)
Chloramphenicol	Cylinder plate	*Sarcina lutea* (Strain known as P.C.I. 1001)
Chlortetracycline	Cylinder plate	*Bacillus pumilus* (N.C.T.C. 8241)
	Turbidimetric	*Micrococcus pyogenes* var. *aureus* (A.T.C.C. 6538-P
Dihydrostreptomycin and Streptomycin	Cylinder plate	*Bacillus subtilis* (N.C.T.C. 8236)
	Turbidimetric	*Klebsiella pneumoniae* (A.T.C.C. 10031)
Erythromycin	Cylinder plate	*Bacillus pumilus* (N.C.T.C. 8241)
Neomycin	Cylinder plate	*Bacillus pumilus* (N.C.T.C. 8241)
	Turbidimetric	*Klebsiella pneumoniae* (A.T.C.C. 10031)
Penicillin	Cylinder plate	*Bacillus subtilis* (N.C.T.C. 8236)
Polymyxin B	Cylinder plate	*Brucella bronchiseptica* (N.C.T.C. 8344)
Tetracycline	Cylinder plate	*Bacillus pumilus* (N.C.T.C. 8241)

* N.C.T.C. = National Collection of Type Cultures.
A.T.C.C. = American Type Culture Collection.

medium are placed in a series of tubes to which suitable amounts of the solution of substance under test are added, the mixtures adjusted to standard volume and incubated. After incubation the bacterial growth in each tube is measured. A calibration curve is prepared by carrying out a similar experiment using a solution of the standard preparation instead of the substance to be assayed and the potency of the unknown is calculated from the readings.

Assessment of bacterial growth may be carried out by the turbidimetric and diffusion methods. The latter when performed by the cup-plate or cylinder-plate modifications affords circular zones of growth or "exhibition"[17] instead of inhibition. By far the most popular methods, however, are acidimetric and these are made possible by the use of micro-organisms, such as strains of Lactobacillus, whose growth is accompanied by the production of lactic acid. Under favourable conditions the amount of lactic acid formed is proportional to the bacterial growth, which may be assessed by titration of the acid. Higher organisms, such as fungi and yeasts are sometimes used, and the dry weight of the organism produced used as the response. Table IV illustrates some of the micro-biological assays which are now established.

Besides vitamins microbiological assays have been employed for the determination of amino acids[15] and trace metals[18], but these are at present little used in pharmaceutical analysis.

Some Application of Microbiological Assays

The moment a new antibiotic is discovered microbiological assay becomes an essential tool for development of the discovery. It is most valuable for following the extraction of the antibiotic from the crude fermentation liquors and for controlling the various stages of purification until it is finally isolated in a chemically pure form; it is indispensable for the assay of its pharmaceutical preparations and for studying their stability and it may be used for investigation of the distribution, destruction and excretion of the product when administered to animals and man

TABLE IV

Vitamin	Method of assay	Test organism*
Vitamin B_1 (Aneurine)	Turbidimetric	*Lactobacillus fermenti* (A.T.C.C. 9338)
Vitamin B_2 (Riboflavine)	Acidimetric	*Lactobacillus casei* (A.T.C.C. 7469)
	Turbidimetric	*Lactobacillus casei* (A.T.C.C. 7469)
Vitamin B_6	Turbidimetric	*Saccharomyces carlsbergensis* (A.T.C.C. 9080)
Vitamin B_{12} (Cyanocobalamin)	Turbidimetric	*Lactobacillus leichmannii* (A.T.C.C. 4797)
Biotin	Acidimetric	*Lactobacillus arabinosus* (A.T.C.C. 8014)
Folic acid	Acidimetric	*Streptococcus faecalis* (A.T.C.C. 8043)
Nicotinic acid	Acidimetric	*Lactobacillus arabinosus* (A.T.C.C. 8014)
Pantothenic acid	Acidimetric	*Lactobacillus arabinosus* (A.T.C.C. 8014)

* A.T.C.C. = American Type Culture Collection.

The technique has played an outstanding part in the development of chemotherapy as an aid to screening antibiotics against a wide spectrum of bacteria. The phenomenal growth of the antibiotics industry during the last ten years has certainly resulted in the diffusion method of assay becoming one of the most widely used analytical tools and hundreds of thousands of such assays must be performed yearly.

The microbiological assay of vitamins has not enjoyed such widespread application but has some notable successes to its credit. That *p*-aminobenzoic acid, inositol, nicotinic acid, pantothenic acid and folic acid were essential for the growth of some micro-organisms was known before their need in animal nutrition was appreciated. The story of liver extract affords a good example of the value of microbiological tests. The introduction of liver therapy for the treatment of pernicious anaemia by Minot and Murphy[19] 30 years ago was followed by the manufacture of liver extracts consisting of crude hygroscopic powders or equally crude liquid preparations. There was no means of assay excepting the haemopoietic response in patients suffering from pernicious anaemia, and, in fact, this was the only guidance available to the many workers who attempted to isolate the active principle. Liver therapy was so effective that suitable patients for testing purposes became scarce and for the next decade progress was extremely slow although purified extracts, suitable for injection, became available and largely replaced oral preparations. In 1940–41 Peterson and co-workers[20,21] reported the presence in liver extract of a factor active in promoting the growth of *Lactobacillus casei* and this led to the employment of microbiological assay as a research tool

in this field. Liver extracts were found to contain several growth stimulating factors for strains of lactobacillus. Shorb[22] reported the presence of a growth factor for *L. lactis* Dorner, and later[23] announced that vitamin B_{12} possessed similar activity of a high order. There is no doubt that this work contributed substantially to the identification of folic acid as the *L. casei* factor and of vitamin B_{12} as the principle active in the treatment of pernicious anaemia. At the same time an accurate assay of liver extracts for vitamin B_{12} was provided.

The discovery that the bacteriostatic action of sulphanilamide is due to its interference with the utilisation of *p*-aminobenzoic acid by susceptible organisms[24] gave rise to the conception of antimetabolites and to a new approach to chemotherapy. It was evident that any substance which prevented the use of an essential growth factor by a pathogenic organism had potentialities as a chemotherapeutic agent. Microbiological assay techniques can readily be applied in the search for antimetabolites. The observation that some pyrimidine derivatives resemble proguanil in being folic acid antagonists[25] led to the suggestion that these compounds might also possess antimalarial activity. Of three hundred compounds examined 2:4-diamino-5-*p*-chlorophenyl-6-ethyl-pyrimidine proved the most active and has become established as an antimalarial drug under the official name of pyrimethamine. Microbiological studies also showed 6-mercaptopurine to be an antagonist of adenine and hypoxanthine[26] and indicated the possibility of using the compound to interfere with the enzyme systems of cells as a basis for the treatment of malignant disease. This work resulted in the use of 6-mercaptopurine in the treatment of chronic myelogenous leukaemia[27,28].

Chromatography

The application of analytical techniques, including *micro*-methods, is very largely dependent upon the successful preliminary separation of the substance to be determined from other ingredients of the sample under examination. Of modern procedures of separation none has made a greater contribution to analytical chemistry than has chromatography, used 50 years ago by the Russian botanist Tswett[29] in his studies on chlorophyll. It was not until 1931, however, when Kuhn and his co-workers[30] used it for the separation of carotenoids that the importance of chromatography became generally recognised. The extreme simplicity of the method may be illustrated by a brief description of Tswett's experiment. A light petroleum extract of green leaves was poured on to the top of a column of powdered calcium carbonate held in a vertical glass tube. By slowly adding light petroleum the extract was washed down the column and as it descended it was observed to separate into a number of different coloured zones. The components could be separated by breaking up the column. Chlorophylls "a" and "b" may be separated in this way although they are very similar in chemical structure; it is this great ease with which closely related compounds are separated that makes chromatography of such value to the analyst. In order to effect separation a laboratory column can only deal with a small load of material.

On the other hand, there is practically no lower limit to the amount of material examined for it is the sensitivity of the method of detection which determines the amount used; this is often in the microgram range. For the purposes of detection chemical, biological and physical methods have been employed and it is true to say that advances in chromatography have gone hand in hand with improved methods of detection. To-day chromatography enjoys an extensive literature which can no longer be followed in detail. The procedures in general use may be classified as (1) adsorption chromatography, (2) ion exchange chromatography, (3) partition chromatography, and (4) gas-liquid chromatography.

Adsorption chromatography. This depends upon the varying adsorptive power of some solids for the substances under examination. Columns have been built of many materials; aluminium oxide, keiselguhr, calcium carbonate, Fuller's earth and magnesium oxide being amongst those most favoured. Early separations were achieved by cutting up the columns between the zones after development but this technique is difficult to carry out with colourless substances and it is more usual to wash the column with pure solvent and fractionally collect the solution, described as the eluate, emerging at the bottom. For analytical purposes it is sometimes convenient to obtain a continuous record by measurement of some physical property, such as the refractive index, of the eluate leaving the column; this is known as frontal analysis. Tiselius[31] found it was advantageous to develop the column with a solution of a substance more strongly adsorbed than those to be separated; this method is known as displacement development. Another device for increasing the efficiency of column separations is gradient elution, in which the composition of the solvent entering the column is gradually changed.

In the pharmaceutical field adsorption chromatography has been used very extensively for the separation of natural products, an outstanding example being the part it played in the discovery of vitamin B_{12}[32,33], which caused such world wide interest in 1948. The use of adsorption chromatography in quantitative analysis is illustrated by the U.S.P. procedure for the assay of digitoxin and the separation of vitamin D_2 from its preparations prior to its colorimetric determination[34].

Ion exchange chromatography. Base exchange, or cationic exchange, has been the subject of extensive investigation since the middle of the nineteenth century and has mostly concerned the zeolites, consisting of aluminium silicates of complex composition. Synthetic zeolites are used in water softening, carried out by filtration of water containing calcium ions through a bed of cationic exchanger, in the sodium form. In this way the calcium ions are replaced by sodium ions. This property of exchangers clearly has analytical potentialities and developments have led to the preparation of a range of synthetic resins, having free acidic or basic groups in their molecular structures. These have wide use in the field of analytical chemistry known as ion exchange chromatography. Columns of ion exchangers are used similarly to those in adsorption chromatography and are employed especially for separating acid and basic components from mixtures. Ion exchangers can be regarded as

ion filters of selective permeability, a cationic exchanger being permeable only to cations, and an anionic exchanger only to anions.

Schultz[35] has reviewed the analytical applications of ion exchangers and a more detailed account has been given by Samuelson[36]. Although the technique has been much used in inorganic analysis, it has also been successfully applied in the organic field which is of particular interest in pharmaceutical analysis. A solution of alkaloidal salt may be passed through a column of cationic exchanger, in the "H" ion form, and the alkaloid held on the column while the eluate contains an equivalent amount of acid which may be titrated. If necessary, the alkaloid may be removed from the column by washing with a suitable solvent containing ammonia; this procedure is valuable for the separation of alkaloids from crude extracts prior to analysis. When vitamin B_1 is determined in natural products it is removed from the preliminary extracts by adsorption on a column of Decalso F, from which it is subsequently recovered in sufficiently pure form to permit its estimation fluorimetrically by the thiochrome reaction.

Partition chromatography. It is well known that when an aqueous solution is shaken with an immiscible solvent until equilibrium is attained the ratio of the concentrations of the solute in the two phases will be a physical constant, known as the partition coefficient of the substance concerned. The difference between the partition coefficients of amino acids was utilised by Martin and Synge[37] to effect their separation when shaken with a battery of extractors containing two immiscible solvents. This procedure with solvent-solvent extractors was further developed by Craig[38] in his counter-current distribution machine. It was found by Martin and Synge[39] that more efficient solvent-solvent extraction could be achieved by use of columns of silica gel holding about 50 per cent of water, placing the sample under test on the column and developing with an immiscible solvent. Other materials, such as starch and cellulose powder, have been used for holding the stationary phase of water and the process has become known as partition chromatography. Later it was discovered that sheets of filter paper[40] could be used to support the stationary phase and this gave rise to paper partition chromatography, which is probably the most versatile method for analytical work on a micro-scale, and it has been used very extensively for the solution of problems in both pharmacy and medicine.

Monographs on paper chromatography have appeared[41], and the technique has been applied in a broad field, including the antibiotics, alkaloids, amino acids, carbohydrates, dyestuffs, glycosides, steroids and vitamins. Much ingenuity has been shown in locating the substances on the paper after development of the chromatogram. Coloured materials present no difficulty; colourless substances are usually located by spraying the paper with a reagent which will afford a coloured reaction product with the compounds under test. Ninhydrin[42] and Dragendorff's[43] reagents are popular sprays for location of amino acids and alkaloids respectively and sometimes, as with ergot alkaloids,[44] examination of the paper in ultra-violet light will reveal the compounds as fluorescent spots. Antibiotics

may be detected by laying the damp paper on an agar plate, seeded with suitable micro-organisms, and subsequent incubation of the plate when zones of inhibition on the agar coincide with the positions of the antibiotics on the paper. Compounds containing radioactive tracers may be detected by bringing the paper in contact with a photographic plate which is then developed. Besides the identification of components of mixtures, paper chromatography has been used for quantitative work by measurement of the intensities of the spots upon the paper or by their removal from the paper by elution with a solvent and use of micro-chemical analysis. Applications in the pharmaceutical field have been extensive and typical examples have been in the study of the glycosides of digitalis[45], the identification of the polymyxins[46], the detection of biologically active fractions of liver extract[47] and the separation and identification of the different penicillins[48]. Perhaps the greatest achievement of paper chromatography, however, was the part played by it in establishing the structure of the insulin molecule[49].

Gas-liquid Chromatography

Martin and his co-workers[50] have extended their work to include liquid-gas systems in which the stationary liquid phase, as a substance of high-boiling point and good stability, is supported on a column of inert solid carrier, such as keiselguhr, and the mobile phase is a gas. The sample under test is blown along the column by an inert gas and the substances, separated according to their volatility, are detected by a suitable device at the end of the column. It has been found advantageous to maintain the column at elevated temperatures. Volatile organic acids and bases[51,52] may be detected by use of an automatic titrimeter at the exit but sensitive physical detectors, giving a continuous record of the thermal conductivity or density of the emergent gases, are now widely used. This new technique has been described as gas-liquid chromatography, vapour-phase chromatography or gas chromatography, and a full description is given in the report of a recent symposium on the topic[53].

As with other types of chromatography, the procedure is of great sensitivity; a sample of no more than a few milligrams is required and as little as 0·1 microgram of test material in 1 ml. of carrier gas can often be detected. There is little doubt that gas chromatography has many analytical advantages in terms of time, expense and information which it makes available and it may well replace analytical distillation. So far the technique has been used little in the pharmaceutical field, although it was recently employed for the detection of impurities in Chloroform B.P.[54]; its use in the examination of essential oils is an obvious application.

Some General Considerations

Any review of modern analytical chemistry would be incomplete without some reference to instrumentation. The thermionic valve and photo-electric cell have extensive applications in the design of scientific instruments and any chemical reaction capable of producing a suitable signal

affords scope for instrumentation, using servo-mechanisms to record chemical and physical changes with a precision unknown 25 years ago.

Full use has been made of instrumental methods in the pharmaceutical industry where the control laboratories employ spectrophotometers, covering the visible, ultra-violet and infra-red regions of the spectrum, pH meters, polarographs, flame photometers, fluorimeters and many other instruments. This has resulted in greatly increased sensitivity and a corresponding decrease in the size of samples examined. For example, when it was standard practice to use visual colorimeters for comparing the colours of solutions the experimental error was of the order of ± 5 per cent; to-day the use of photoelectric spectrophotometers has reduced the error of such determinations to $\pm 0{\cdot}1$ per cent. Radioactive elements have been used widely as tracers for metabolic studies in biochemistry and medicine[55] but, so far, the technique has been little used in pharmaceutical analysis. It is to be noted, however, that solutions of Sodium Radio Iodide (^{131}I) and Sodium Radio Phosphate (^{32}P), requiring the use of a Geiger-Muller counter for their standardisation, are included in the B.P. 1958.

Microchemical analysis, microbiological assay and chromatography, in conjunction with instrumentation, embrace much of modern analytical chemistry. If analysts of a past generation laid the foundations of pharmaceutical analysis when the general analytical method was "dry, ignite and weigh", the analysts of to-day can justly claim that they have not missed their opportunity to apply modern analytical developments to the solution of pharmaceutical problems. The pharmaceutical industry consists essentially of research leading to the discovery of a new drug, the purchase of raw materials needed for its manufacture, pharmaceutical development resulting in its satisfactory presentation, the production of the product and its preparations, advertising its availability and finally its commercial distribution. In the pharmaceutical field no organisation can operate successfully without the support afforded by the analytical chemist; no research worker, no production manager, no buyer, no publicity manager and no salesman could face his task to-day without the backing of a reliable analytical laboratory. That this Conference is fully aware of the importance of analytical chemistry in pharmacy I have no doubt; this has been shown by your election of an analyst to be your chairman this year. I do not feel this is an exclusive personal honour but prefer to consider it as a tribute to that army of analysts, particularly the young laboratory assistants upon whom the burden of much routine work falls, who ceaselessly labour in the control laboratories of our pharmaceutical factories. It is they who safeguard the interests of the prescriber, the pharmacist and the patient and do so much to keep British products in the vanguard of pharmaceutical progress.

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SYMPOSIUM

THE EVALUATION OF NEW DRUGS

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The development of synthetic organic chemistry has brought with it profound changes in our mode of living, not least in the field of medicine. A glance at the 1958 British Pharmacopoeia, as compared with the first edition of a hundred years ago, serves to illustrate this point. An additional striking feature is the diversity of chemical types that find employment in this way. There is no such thing as a favoured molecular species, and although this widens the scope for investigation, it vastly increases the task of those concerned with the search for new drugs. Fortunately, parallel advances in experimental biology and scientific medicine have aided the process of selection, and it is of interest to survey the current position.

Basically, the discovery of a new therapeutic agent, using this term in its broadest sense, has three distinct phases:—

1. The discerning of a potentially useful biological effect in a chemical compound, either synthetic or natural in origin.

2. The determination of the comparative safety of the compound using laboratory animals.

3. The tentative trial of the compound in man or domestic animals.

This scheme is an over-simplification, but those engaged in this kind of work will identify each of these stages with the three major obstacles that have to be surmounted before success can ultimately be achieved. Other disciplines such as pharmacy and biochemistry all have important contributions to make; the former in presenting the drug in its safest, most effective, and most convenient form, and the latter in arriving at a mode of action, perhaps thereby aiding a more intelligent application.

It is possible to formulate an idealised flow-sheet to cover the discovery and development of a new drug, but in practice very few examples taken from the vast number of agents now available will be found to have conformed to it. The pattern, however, is reasonably standard for the progression of a new drug in the immediate pre-clinical stages, although the final decision still depends heavily on human judgment and on a compromise between extreme caution, and the natural desire to bring the work as rapidly as possible to a useful conclusion. In this communication it is proposed to outline and discuss, sometimes using specific examples, as many as possible of the factors which have to be considered.

The discovery of drugs is not a monopoly of any one institutional or industrial section of the community. Instances can be quoted which had

their beginnings in all manner of university and hospital laboratories. But in recent years the main concentration of effort has been in organisations based on the industrial pattern, and although it might be said that the authors are biased in this matter, there is every reason to suppose that research carried out under these circumstances is conducted with at least the maximum economy and speed, if only because of the highly competitive nature of the pharmaceutical industry. Be that as it may, the course of events when the main function of the unit is the search for new therapeutic agents is much the same, wherever they may occur. This is true whether the end product is an antibiotic or synthetic substance, although this paper will deal almost entirely with the latter.

The Chemico-Biological Approach

At the outset, the organic chemist must have a chemical lead of some kind. This might be provided by knowledge, possibly incomplete, of the structure of a substance showing something of the biological effect he is looking for. The prototype molecule may have arisen from a chance observation in the laboratory or clinic, or the behaviour in a given assay of a compound prepared for another purpose, not necessarily even therapeutic, or it might be that of a substance contained in and isolated from a native remedy. Alternatively, the molecule could have been designed on a more rational basis in an attempt to disorganise or modify the function of an essential chemical factor within the target cell, be the latter parasitic, or a normal component of the animal body. Given such a lead, the chemist can begin almost immediately to devise and then synthetise potentially improved structural variants of the prototype. If constructive progress is to be made, however, it will be necessary to have the new compounds assayed by a test sufficiently precise to show up small changes in biological activity in a statistically significant manner. The development of such a test is the prime function of the biologist member of the team. It may take the form at first of an assay *in vitro*, but it is essential to augment or replace such a method as soon as possible by a replica of the particular condition under investigation in laboratory animals, the smaller the better, thus enabling the maximum experimentation to be made with the quantity of material available. In practice, the evolution of the biological assay method is usually the most time-consuming and the most expensive aspect of chemotherapeutic research. The problem is at its simplest when it concerns infectious diseases caused by micro-organisms and particularly those that can be cultured *in vitro*. The initial screening can then be directly on the causative organism, and a few mg. of the agent is all that is required. But even so, there are uncertainties apparent which make it undesirable to place complete faith in such a procedure. For example, what level of growth-inhibitory concentration should be used to define positive activity? It would be unwise to fix this too rigidly since if the figure is to be related to the limiting tissue concentrations thought to be essential for a corresponding therapeutic effect in the infected animal, then the chemical and physical properties of each substance will need to be taken into account, because

these, at any rate in part, govern the absorption and distribution characteristics *in vivo*. Likewise it is sometimes necessary to distinguish between mere inhibition of growth and an effect leading to the actual death of the disease incitant. This in turn involves the contribution that can be expected from the defence mechanisms of the host animal, which may differ from species to species. Then the growth of some micro-organisms is more easily suppressed than others. For example, the tubercle bacillus will cease to multiply at concentrations measured in a few parts per million, of a large number of simple phenols and aromatic amines, especially those that oxidise with facility to quinonoid structures. Yet not one of these substances is known to be effective *in vivo*. There is good reason to suppose that the tubercle bacillus in the infected animal differs from the same strain in the culture tube, and amongst other things in its response to the action of drugs[1]. Finally, an assay *in vitro* cannot take account of metabolism of the test substance in the animal body. It is well-known that the sulphonamide drugs would not have been discovered in the way that they were if Domagk had not proceeded early to an examination in infected mice of the azo precursors, themselves having only marginal action *in vitro*. In this sense, the administration of any single organic compound to an infected animal can be regarded as an experiment made in fact with a number of related substances.

Some infective disease conditions can only be studied away from the animal host with great difficulty. For example, it is not possible to cultivate malaria parasites or trypanosomes *in vitro*, although it has been found possible under carefully controlled conditions to maintain the viability of these protozoa in isolation for a sufficient length of time to study the effect of drug action upon them. In at least one case, that of the antimalarial drug proguanil, such a technique was of value in directing attention to the formation *in vivo* of an active metabolite[2]. Fortunately from the point of view of facilitating the chemotherapeutic study of these diseases, elegant and comparatively simple methods have been worked out for producing controlled infections in laboratory animals.

Perhaps the most difficult of the infective diseases to study experimentally are those due to viruses[3]. In the first place, inactivation of virus *in vitro* is far too easily achieved by a variety of agents to have any significance as a method for sorting drugs. Experiments using tissue cultures have been of greater value in elucidating the mechanism of drug action but have not helped in developing any new effective agents, due mainly to difficulties associated with technique. Many viruses will multiply in contact with the chorio-allantoic membrane of the chick embryo and the influence of chemical compounds on the process can be measured either by introducing them on to the chorio-allantois or into the yolk-sac. Both routes are open to criticism, but the latter can be regarded as approximating to a therapeutic assay *in vivo*. It has been common experience, however, that results obtained in tissue culture and chick embryo are rarely transferable to animals such as the mouse, or even to the hatched chick, from which it follows that for progress in the chemotherapy of the virus diseases direct medication in an infected animal

is necessary. This in turn brings its own snags and complications, not least those arising from the lower rate of virus multiplication observed in animals made weak or sickly from toxic drug action. Research in this field is extremely costly, and the continued insusceptibility to drug action which now seems characteristic of these diseases is in itself a source of discouragement to all but the hardier spirits. Nevertheless, it does go on.

Until perhaps the middle of the last decade, the bulk of chemotherapeutic research effort, for a number of reasons, was directed towards the diseases of micro-organismal origin of the type that have just been considered. Although there are notable exceptions, intensive work in laboratory animals on non-infectious diseases, for example those of organ-dysfunction, has only begun in comparatively recent times. This has required an almost complete re-orientation of outlook in many of the longer-established research units and with the new entrants into the field, has in turn been reflected in the current shortage of pharmacologists who are now urgently needed in increasing numbers for this kind of investigation. This statement is not so flippant as it might seem, because as may be by now apparent, the limiting factor in the search for new drugs is rarely the ability of chemists to produce likely candidates for examination, but more often the absence of a means of testing them. By its very nature, organic disease is often difficult to establish in a test species bred to be intrinsically healthy, and when it concerns the higher functions such as the central nervous system it may not be possible to parallel experimentally the stresses of the higher primates. Thus the very great interest of the present time in sedatives and the so-called tranquillisers has called forth a tremendous amount of work on all manner of ingenious test procedures. Similarly, although a wide range of anti-epileptic drugs is available to medicine today, there is still no experimental method which will distinguish with precision between substances acting on the two principal forms of epilepsy, and final judgment has to await the outcome of clinical trial. Malignant disease is also represented by a great variety of laboratory conditions, from the apparently spontaneous development of tumours in the mammae of a particular strain of mice, to the chemically induced, and finally to those grown as the result of subcutaneous implant. Yet no single experimental technique can be relied upon to screen compounds likely to inhibit the growth of a pre-defined malignant condition in man, and in practice a whole battery of methods is frequently used in the cancer research institutions to ensure detection of even the faintest glimmer of activity.

The Biochemist

So far, chemotherapeutic research has been discussed only in its most elementary form of the chemist synthesising a potential drug and the biologist subjecting it to assay. Given ingenuity in devising a hypothesis relating chemical structure to biological activity, discovery can come quite soon in this way and with great economy of effort. Even so, it is the experience of many groups of workers that only one compound in every

thousand synthesised is likely to be even considered as a candidate for clinical trial, and in a well-equipped unit with heavy overhead and maintenance charges the total average cost of preparing and assaying each separate substance can be as high as £200. Expense apart, there is least intellectual satisfaction in proceeding solely along semi-empirical lines even when the so-called rational approach has been attempted. The latter can rarely take count of the differential activity required in chemotherapy between host and parasitic cells which cannot be distinguished biochemically on present-day knowledge except in the crudest of terms. Much less can it explain the catastrophic changes in biological activity that sometimes follow even the subtlest variation in chemical structure, such for example as the almost complete disappearance of trypanocidal activity which results when the two methyl groups, seemingly insignificant in polarity and size, are removed from the large and highly polar molecule of suramin[4]. It follows therefore that the mode of action of most drugs, however evolved, is at best only partially understood at the time of their discovery. It is primarily the task of the biochemist to make good this deficiency, and in so doing to provide background knowledge which might lead to the synthesis of more effective agents.

Conversely, the biochemist is frequently engaged to study a disease as a preliminary to the commencement of chemotherapeutic research proper, in order to provide a starting point for the chemist and the biologist. This notion was behind the work which lead to the isolation and the elucidation of the structure of Mycobactin, a growth factor for the causative organism of Johne's disease, and which was to be found only in acid-fast bacteria[5]. The plan was to devise specific inhibitors which should then have been peculiarly antituberculous or antileprotic agents. Unfortunately, the molecule was so complex ($C_{47}H_{75}O_{10}N_5$) that the chemists found it difficult to devise potential antagonists even on paper, and although many hundreds of compounds were ultimately prepared, not one exhibited antituberculous activity *in vivo*. It is also the biochemist who is usually called upon to study the distribution and fate of drugs in the animal body. Work of this nature has perhaps not been pursued with sufficient intensity in the past, but it is absolutely essential if chemotherapy is to be established upon a more scientific basis. Most of the advocates of the rational approach have in mind only the relationship between the structure of the drug molecule and its action on an isolated cell or an enzyme system contained therein. It is not always appreciated that a therapeutic agent can be described only thus when it is able to exert its influence on the target cell *within the tissues of a living animal host*. Under these circumstances, complex considerations of absorption, distribution, excretion and metabolism, have to be taken account of. We know far less about the relation between chemical structure and these properties (which differ anyhow from species to species) than is known about the association of structure with action on individual cell systems. Until this deficiency is made good, the ultimate design of potential drugs, particularly for systemic medication, will continue to involve a large element of uncertainty.

The Pharmacist

While the potential drug is still in the early laboratory phase, only scant attention will have been given to the form of presentation to the experimental animal. If it is soluble in water, all well and good, while if sparingly soluble it will in all probability have been put up for test as a crude suspension prepared by a standard procedure in a rudimentary type of ball-mill. Since it will be used without delay, little thought need be paid to physical or chemical stability, while often the matter of sterility can be largely ignored, even when subcutaneous or intraperitoneal injection is involved. So soon, however, as interest in the drug reaches the point where clinical or veterinary field trials seem a likely possibility, it is desirable to think in terms of the ultimate forms in which it will be administered. Neglect to do this at an early date can cause delays at later and more critical stages. This is an important function of the pharmacist in the total research effort, and ideally he should begin working and thinking on these matters alongside the chemist and biologist from the time of first indication of promise. He must learn all he can about stability, particularly in the face of the possible need to provide a sterile preparation, and indeed as a guide to the treatment that can in general be meted out to the compound during the manufacture of formulates. He will almost certainly have to assume responsibility for analytical control, and this in turn calls for detailed knowledge of the chemistry of the substance and also of the preparative route so that he can guard against the carry-over of possible toxic intermediates into the final product. Needless to say, analytical procedures must relate to the formulations as well as to the pure drug. Above all, in devising likely formulations he must consider the feasibility of ultimate manufacture in the factory and work out processes that lend themselves to the necessary scaling-up. Suspensions that form a clay-like deposit on storage in bulk or in vials, powders that cannot be dispensed in automatic filling machines, cream preparations that stop the stirrer, and tablets which fragment when handled by the hundredweight, are not popular with production managers. All these are problems which invariably come the way of the pharmacist to solve.

Process Development

In building up a complete, even if sketchy picture of the search for a new drug, it must not be overlooked that works manufacture of the bulk chemical will eventually be required. Initially, the organic chemist will have aimed at the synthesis of the required compound without regard to the feasibility of the processes for large-scale production. Rarely are quantities greater than 5 g. needed for the earliest biological experiments, and in the laboratory he can indulge in Grignard reactions and ether extractions to his heart's content. But the mode of preparation he has employed will often be quite impracticable, and costly beyond reason, for translation to a manufacturing unit. It may not suffice even for the production of the modest few pounds needed for extended toxicity tests and early clinical trial. It is at this point that the process chemists begin

their work, and in well-planned development they can combine the study of alternative manufacturing routes with the provision of useful quantities of the drug for further therapeutic experiments as well as for tentative formulation research. Even so, it is common experience that manufacturing efficiency in the early years of a new drug is comparatively low, and the high initial cost is often a reflection of this state of affairs rather than a deliberate attempt on the part of the manufacturer to charge as much as possible for his product. It also frequently happens that despite intensive and costly process work, a given route has later to be abandoned when it is found to be intrinsically uneconomic, and a fresh start has to be made. This in turn may call for the design and installation of entirely new chemical plant, causing further delay and expense.

Pre-Clinical Requirements

When an active drug has been selected, the next problem is to find out whether it is safe to give to man. The final decision to give a new drug to a human being is always a very difficult one to make. In spite of tests on experimental animals, it is impossible to be quite sure that the human subject will react in the same way; there is always the element of chance, and the possibility that the drug may have unforeseen toxic action. In order to minimise the chance of an unpleasant surprise of this kind, it is essential to make very full toxicity tests in the laboratory. To issue a drug for the treatment of human disease without this safeguard is quite indefensible. It will be remembered that last December[6], a Paris Court awarded 643 m. francs to people who were incapacitated, and to the families of the 102 persons who died after taking an organic tin compound for the treatment of boils. This has been described as the worst disaster ever caused by a drug and it happened only a year ago. Had this drug been given a full pharmacological examination it is doubtful if it would have ever reached the stage of clinical trial[7].

Toxicity Tests in the Laboratory

In the course of tests for activity a rough idea of the amount of drug which is safe to give to animals will have been obtained. It is usual to determine the LD50 of the drug (the dose which kills half of a group of animals) when given by mouth and when injected. Mice are often used for the determination of acute toxicity in this way. This is not sufficient; the drug must be given to animals repeatedly in order to study the effects on their organs when exposed continuously to the drug for a period of weeks or months. The growth rates of treated animals are compared with those of untreated controls, and the blood is examined microscopically and biochemically to detect any effect on the blood-forming tissue and the endocrine glands. At the end of the test the animals are killed and the organs examined histologically. A chronic toxicity test of this kind usually reveals actions which are not seen in animals which are acutely poisoned. The doses given must be high and when the toxic level is found, experiments must be made to determine the safe one.

Rats are also used for preliminary tests of this kind. It is essential to use several species of animal because different species often have different degrees of tolerance to a drug. It is hardly necessary to be reminded of the classical example of the rabbit, which can live on belladonna leaves, because it is insensitive to the poisonous effects of atropine. It would not be satisfactory to use the rabbit alone as a guide to the safety of belladonna alkaloids in man.

It is of value to know the toxic effects not only to rodents, but also to carnivores (dogs and cats), and the final assessment of toxicity must often be made in monkeys, which bear the greatest resemblance to man in their reactions and metabolic processes. Attention must be paid to the toxic effects in the most susceptible species, and when the trial dose for man is chosen, it must be a safe dose for this species. All toxicity tests should be planned and performed in collaboration with the pathologist who is responsible for the examination of the animals and their tissues at the end of the experiment. Often it is possible to detect clinical signs which may be of importance when the drug is tried in man.

All this is, of course, very time-consuming and very expensive. The chemist, the pharmacologist and the manufacturing company are usually all anxious to see whether the drug works in man, because if it does not, most of the work may have been in vain. Nevertheless, the tests must be done.

Clinical Trials

It is reasonable to say that it is legitimate to try a drug in man when the pharmacologist, the pathologist and the chemist, having examined the results of all the tests, are willing to take a therapeutic dose themselves. This criterion does not, however, always apply. If a drug is expected to have an ameliorating effect in diseases such as cancer, which advances inevitably to death, it is reasonable to use something more toxic than would be admissible for the treatment of headaches or malaria.

The first dose of a new drug is always attended with some anxiety. There is always someone who has an extreme sensitivity to any particular drug and it is unfortunate if he happens to be one of the few people chosen to take the first dose of the new drug. Useful remedies have been discarded more than once as a result of misfortunes of this kind. An example of a drug which weathered the storm is chloramphenicol, once said by the medical journals to be safe only for use in typhoid fever (which may be fatal), but now prescribed for much less serious ailments without extensive toxic reaction. An example of a drug which did not stand up to the test in man is one of the pyrimidine antimalarials, which was the precursor of proguanil. It unexpectedly produced severe frontal headaches. This effect could not have been forecast by any of the extensive animal tests to which it had been subjected. The effects of tranquillising drugs, which depend for their activity on their selective effects on parts of the human brain, cannot be finally assessed in any other animal than man. Another unexpected casualty among new drugs was the dye, methylene violet, discovered by Hawking to be effective on

filiarial infections in cotton rats. When tried in Africa it was found to cause the nails of the toes and fingers of the patients to fall out. Fortunately, the nails grew again. Because he had done a great deal of laboratory work on these dyes, Hawking spoke very forcibly of the necessity for early clinical trials in man[8].

As indicated earlier in this paper, every opportunity must be taken to gain knowledge of the way in which a new drug is absorbed and excreted, and before the first doses are given to man, it is desirable to have a method for determining the drug or its breakdown products in the blood and the urine. These methods will have already been worked out for use in experimental animals, but it must be remembered that the doses given to animals are usually large; a method which is suitable for the detection of the drug in a monkey which has been given doses close to the toxic range may be not sufficiently sensitive to detect the small quantities to be found in man after a small therapeutic dose. It is better to be prepared for this, if possible, so that the maximum information may be gained from the first human subjects.

Clinical trials are not always easy to arrange. There are a few hospitals, research institutes and university departments which have specialised in the study of new drugs of particular types, such as hypotensives, local anaesthetics, tranquillisers and drugs for treating neoplastic diseases, and these are usually co-operative. However, it is becoming increasingly difficult to persuade such people to try a drug which can be regarded only as a possible improvement on drugs which are already in use and are reasonably effective. This is a pity because advantage should be taken of any improvement in a drug, such as greater specificity of action, lower toxicity or less liability to produce unpleasant side effects; the proper assessment of such an improved substance requires careful and painstaking work and a great deal of experience. Conditions are even more difficult when a drug is to be used for treating diseases to be found only in the tropics. Medical Officers in tropical countries are always exceedingly overworked; with the best will in the world it is often quite impossible for them to give the time and attention that is necessary for clinical trials of a new drug. There is only one way in which these difficulties may be resolved satisfactorily and that is for a pharmacologist who has been associated with the development of the drug from the start, and who knows from his observations in the laboratory its possibilities and its short-comings and the toxic effects which it is likely to produce, to work with the clinicians who are conducting the trial. If such an arrangement can be made, this kind of team work gives the best chance of discovering the truth about a drug.

The ease with which a clinical trial can be conducted depends upon the nature of the pharmacological or chemotherapeutic properties of the new drug. The simplest tests are those which can be made on groups of volunteers—university students or laboratory workers. Professor Keele at Middlesex Hospital has for several years investigated the relative potency of analgesics by measuring their effects on the pain produced by muscular movements of the arm when the blood supply is interrupted

by a sphygmomanometer cuff[9]. Professor Bain and his colleages at Leeds have made experiments on student volunteers with new local anaesthetics and antihistamine drugs[10]. There is usually no difficulty in obtaining full co-operation from medical students in these investigations, when their purpose and value are explained.

Another fairly simple type of trial to arrange is one upon an infection that is not dangerous to life, provided that the tests for toxicity of the new drug have been adequate. An example is provided by the investigations which have been made at Porton by Andrewes on the effect of drugs on the common cold, using experimentally infected volunteers[11]—an ideal arrangement in which the variables can be controlled with the same precision as in laboratory experiments on experimentally infected animals. However, it is important not to generalise from a single example of a type of drug tried clinically. Andrewes tested an antihistaminic drug on the common cold which happened to have very low activity as an antihistamine. It is possible that with a more potent member of the series, he might have obtained a more promising result[12].

Further examples are the experiments made in East Africa upon volunteers infected experimentally with known strains of the malaria parasite[13], and in West Africa by Bruce-Chwatt on natural malaria infections[14]. In the U.S.A. valuable work was done upon the activity of antimalarial drugs in the State prisons, where volunteers were infected with known strains of the parasite and treated with drugs at different stages of the infection[15]. These experiments, perhaps more than any others on malaria, have given us an insight into the way the drugs act on the tissue forms and the blood forms of the parasite. British Law does not allow the occupants of H.M. Prisons to volunteer for useful work of this kind.

A good deal of thought and enquiry is necessary to determine the best place for carrying out a trial upon naturally infected people. A place must be chosen in which the incidence of the infection is high, otherwise much time and effort may be expended in the examination and rejection of uninfected individuals. For example, a tropical city such as Bathurst in the Gambia is ideal for investigating the action of drugs on roundworms[16]. Here about 60 per cent of schoolchildren have *Ascaris* eggs in their stools on the first examination and there are relatively few other worms present. It does not take long in an area of this kind to accumulate a useful number of subjects for a clinical trial. For threadworms, a children's hospital is the obvious place for trials, and for whipworms, an institution for mentally retarded children, where the habits of the inmates ensure the regular transmission of this parasite. A hospital, school, or institution is always the place of choice for conducting a trial because it is possible there to keep a check on the recipients of the drug and to examine them again days or weeks later to see if the parasite has been eradicated or reduced in numbers.

At the other extreme of human afflictions, the trial of a drug against cancer is relatively easy to arrange because many people are anxious to make advances in this difficult field. Here we are faced with a progressive disease which usually has a fatal outcome, and there are very few drugs

to use against it. The nature of the disease is such that a drug which arrests the disorderly division of cells in the neoplasm is likely also to affect the orderly multiplication of the normal cells of the host. Present-day drugs used in neoplastic disease are therefore by nature poisonous, and the margin of safety is small. Great experience and skill are necessary in those who carry out trials of drugs against neoplasms. The selection of suitable patients for the treatment is not easy; it is necessary to decide whether surgery or radiotherapy would be of more benefit to the patient.

Even greater difficulties are encountered when clinical trials are required in diseases which may be dangerous if left to progress and which already have methods of treatment that are known to be effective. It is inhuman and quite indefensible to allow the condition of a patient to deteriorate during the trial of a new remedy, so that he stands a lesser chance of recovery when treated with drugs of established value. In this connexion, the trials of antitubercular drugs organised a few years ago by the Medical Research Council are models of careful planning and selection of patients[17]. Unfortunately, such resources are seldom available for clinical trials, and the best that can be done is for us to learn from the reports the importance of limiting the variables as much as possible and of recording all relevant information in an experiment which is so designed that statistical methods can be applied to the results.

The overriding consideration for those who are engaged in trials which use man as the experimental animal, must be to take every possible safeguard against causing harm by our activities, and to hope that some good may ensue.

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DISCUSSION

In presenting their paper the AUTHORS commented on some current problems.

DR. ROSE outlined the development of a new antituberculosis substance from ethyl Bunte's salt. Its hydrolysis product, ethyl mercaptan, was a potent antituberculosis agent but had many undesirable properties. The

corresponding disulphide had more favourable properties but was not suitable for clinical use. A non-toxic substance which liberated ethyl mercaptan in the tissues was sought. Eventually the bisethylthiol ester of *iso*phthalic acid (Compound 15688, Etisul) was chosen. It was found to be potent by the percutaneous route, but like ethyl mercaptan and the disulphide had no *in vitro* activity. Attempts to elucidate the mode of action of these compounds had not yielded a great deal of information. The active metabolite was still not known, but he hoped that this unknown substance would come to light and that it was not a mercaptan. Methyl mercaptan and methyl thiobenzoate antagonised the antituberculosis effects of these compounds. Compound 15688 was going to clinical trial in both tuberculosis and leprosy, and would be administered percutaneously.

DR. GOODWIN said he wished to emphasise the importance of toxicity tests, and that the metabolism of a drug should be known before it was tried in man. This was often difficult because drugs now being made approached in structure the metabolites found normally in the body. Adequate controls were necessary in clinical trials, particularly with a drug such as an analgesic. In the tropics a new compound would be tested in well organised communities where it was fairly easy to keep track of the patients, but this was very different from what often happened with a drug given to people in African villages or even in some African hospitals. In such places there must be the shortest possible period between the first examination or treatment and the follow-up examination —often much shorter than was desirable. This meant that a drug for use in tropical areas should if possible be given as a single dose. A new drug may show no undesirable side effects on Europeans, but in a country where the standard of nutrition was low, and the light intensity and the humidity high, unexpected side effects might be seen.

DR. G. F. SOMERS (Liverpool). The synthetic chemist usually began with preconceived ideas of chemical structure and possible relationships to drug action. In any screening programme the search should be wide, otherwise important discoveries were likely to be missed. He wished to underline the importance of the statistician's contribution to this work.

DR. ROSE. His experience was that the active compound was usually found by the man who was looking for it. It was not altogether unexpected that a substance designed for one condition might be more active against another. Chemotherapy provided many examples.

MR. S. G. E. STEVENS (London). There was increasing interest in the effects of metabolites of drugs. In the evaluation of a drug, ought one to consider the long-term effect of such metabolites? In certain parts of America the inmates of State prisons were occasionally used for such tests. Would the Authors be prepared to persuade the Government to allow some work of this sort in this country?

DR. GOODWIN. The metabolic products were very important. One example was proguanil, which had to be metabolised in the body before it

worked as an antimalarial. Similarly, chloroquine was stored in the liver in large quantities. He did not think British law would permit trials on prisoner volunteers.

DR. J. M. ROWSON (Ibadan). The average African appeared to carry half-a-dozen diseases in his blood stream. Hospitals in the bush were usually primitive, and contrasted with the few good ones. There seemed to be a great future for native drugs; in Nigeria hundreds awaited investigation. The stability of pharmaceutical preparations in the tropics needed more control. Packs often deteriorated after only a few months.

DR. ROSE. His own interest in native remedies was to obtain a chemical lead for future synthetic work.

DR. GOODWIN. The place for research on African drugs was in Africa. The universities there had good botanical and pharmacological departments. The administration of drugs to queues of patients in the tropics was a very real problem. A drug which was injected pleased the African very much, but it was a nuisance to the pharmaceutical department. Something given by mouth was therefore very much better, but a tablet was a valuable commodity, and moreover it was not easy for everyone to swallow tablets. Something which was taken as a draught was best for a long line of people.

MR. G. R. WILKINSON (London) and MR. H. J. BRAGG (Folkestone) stressed the need to bring the pharmacist into the research team at an early stage in the development of new compounds.

DR. ROSE. The organic chemist, pharmacologist and biologist should work hand-in-glove with the pharmacist from the very beginning. It might be that the organic chemist, responsible for synthesising the potential new drug, could make changes to its structure to meet pharmaceutical problems.

MR. J. B. LLOYD (Manchester). Had the Authors considered, during this present period of very intense activity in synthetic drugs, having some central bureau for the organisation of clinical trials? Another problem was the legal aspect of administering an inert substance to a patient who because the trials are blind could not be asked to give his consent.

DR. GOODWIN. The Medical Research Council undertook limited special trials. It was usually possible to obtain the patient's consent to receive a new drug. It was certainly possible to obtain the consent of medical students, for example in analgesic or toxicity tests.

DR. ROSE. A clinical trial should be conducted close to the laboratories, because the absorption and distribution of the drug could then be studied if necessary by special techniques.

MR. A. BRAGG (Liverpool). Long term stability tests should be carried out in parallel with long term toxicity tests both on the drug and its formulations.

DR. ROSE. Accelerated ageing tests cannot entirely replace actual storage experiments.

MR. N. HERDMAN (Edinburgh). Would the authors enlarge on the possibility of the increased use of precursors in controlled fermentation or in fertilisers and soils to achieve controlled biosynthesis in plants and animals?

DR. ROSE. What was needed was fundamental study of the way in which Nature built up molecules. For instance, one could feed ^{14}C to a plant.

DR. R. L. BLYTHE (Philadelphia). The procedures for testing new drugs in Britain and America were similar.

DR. ROSE. There was a tendency for the duration of toxicity tests to be increased. If, during the histo-pathological examination, there was any sign of cytotoxicity, tests must be conducted for the lifetime of the animal species used.

PROFESSOR E. SHOTTON (London). The main function of the pharmacist in this work was formulation. If he was an analyst he was not functioning strictly as a pharmacist. Much more work needed to be done on preservation in order to correlate the physical properties of the preservatives with those of the preparations.

DR. ROSE. What the Authors had stated in the Paper was that the pharmacist would almost certainly have to assume responsibility for analytical control but not necessarily have to do the work itself. He maintained that the pharmacist did have the final responsibility for acceptance.

MR. B. B. NEWBOULD (Sheffield). What function should the national Press have in informing the general public of recent advances in chemotherapy? Should communications be issued by or through any particular body to ensure accuracy?

DR. ROSE. So often the lay Press had the wrong end of a story. Sometimes it was the fault of the scientists, who stressed a point which they thought was interesting but which was not the point which interested the lay public. There was no doubt about the interest of the public in discovering new drugs.

DR. GOODWIN. The more reputable papers and journals often carried good scientific articles in which a balanced view was presented.

DR. P. T. CHARLTON (Nottingham). Many micro-organisms could split the S—S link of disulphides, and among the products of metabolism were found considerable quantities of methylated compounds—the methyl alkyl sulphides. There must be a close connection between metabolism in the animal body and the activities of these sulphur compounds against micro-organisms in that body. It seemed possible that methyl mercaptan acted as an antagonist to ethyl mercaptan because it was being used as a more normal metabolite. Micro-organisms could also exert a methylating action on the other compounds in that group, selenium and tellurium. Had Dr. Rose considered using selenium and tellurium compounds in a similar way?

DR. ROSE. It had been their experience that selenium compounds were highly toxic. They had not tried replacing sulphur with selenium in their active substance.

MR. R. E. LISTER (Edinburgh). What was the ethical position in using placebos in controlled experiments and withholding drugs of known value from suffering subjects?

DR. GOODWIN. The suffering of many who required analgesics was of psychological and not organic origin. In such cases a placebo would often do as much good as something more potent and more poisonous. It was extremely important not to withhold adequate treatment from patients, and it was the business of those conducting clinical trials to see that the patient did not suffer as the result of the trial of a new drug. Any trial must not continue for so long that the patient forfeited his chance of benefiting from already established medicine.

MR. C. F. ABBOTT (Liverpool). Industrial pharmacists used kinetic studies to predict the life of a product and to avoid the irksome two-years' delay.

DR. ROSE agreed, but there might be a chain reaction, with several decomposition products. It was often difficult to forecast exactly what the position would be at the end of two years on the basis of a three months' experiment.

MR. V. REED (London). Was the antidote to a new drug always available?

DR. ROSE. The best antidote to a new drug which proved toxic was to withdraw it.

DR. GOODWIN. If the drug was an antimetabolite, they could always have the metabolite available.

DR. ROSE. This underlines the importance of the biochemical study of the action of the drug, which might well suggest a substance which would antagonise the drug and act as an antidote.

MR. S. DURHAM (Sheffield). To what extent did the animals used in the preliminary trials reproduce conditions found in humans? What was the value of clinical tests on the patients of general practitioners?

DR. GOODWIN. The mouse, the rat, the guinea pig and the monkey were, on the whole, more different from man than man in England was different from man in the tropics. A clinical test by general practitioners would have to be carefully arranged with the College of General Practitioners.

MR. J. C. HANBURY (Ware). A great many scientifically trained students were going to the less developed parts of the world, and one of the best services such people could do for the advancement of medicine was to make a serious, scientific appraisal of the traditional native medicines. One of the greatest difficulties to be faced in the future was the provision of adequate facilities and material for the clinical evaluation of the very large number of drugs which would come into service in the next few years.

MR. T. D. WHITTET (London). Statistics issued by the Home Office about experiments on animals were somewhat misleading. By far the majority were simple injections which caused no pain. There could be an improvement in public relations by stressing the simple nature of these experiments.

MR. D. F. SMITH (Bournemouth). Did the Authors consider the present methods of clinical trial adequate, or was it possible for material to be launched on to the market before it had been adequately tested by even the present standards?

DR. GOODWIN. The development of drugs was a continuing process. Much work might go into a compound which was hailed as useful and important, but it might be replaced by something more active as a result of work going on elsewhere. A certain amount of material did reach the market without adequate clinical trial.

SCIENCE PAPERS AND DISCUSSIONS

THE BASIS FOR "SUFFICIENT OF A SUITABLE BACTERIOSTATIC" IN INJECTIONS

BY G. SYKES

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In the light of the relative lethal and inhibitory properties of several known bacteriostats, including those recommended in the B.P., along with some observations on their partitioning ratios between rubber and water, the requirements for "sufficient of a suitable bacteriostatic" are discussed, and various criteria are suggested. Attention is drawn particularly to the narrowness of the margin between effective lethal concentration and minimum inhibitory concentration of many bacteriostats and to the consequent need to prevent their loss by absorption into rubber, or through any other cause. Activity against mould spores as well as against resistant vegetative bacteria is also desirable.

THE primary reason for including bacteriostatic substances in parenteral preparations dispensed in multidose containers is to ensure that contaminating organisms, introduced accidentally whilst withdrawing successive doses, shall not be able to proliferate and so cause subsequent damage to the medicament or, even worse, give rise to an infection in the patient. There are, of course, other possible sources of contamination, for instance during the preparation or storage of the injection, but adequate safeguards and controls are now available to prevent such occurrences. The British Pharmacopoeia requires that all such preparations in multidose containers shall contain "sufficient of a suitable bacteriostatic to prevent the growth of micro-organisms," and it cites as examples "Phenol, 0·5 per cent w/v; Cresol, 0·3 per cent w/v; Chlorbutol, 0·5 per cent w/v; Chlorocresol, 0·1 per cent w/v; Phenylmercuric nitrate, 0·001 per cent w/v." But these solutions vary considerably in their antimicrobial properties—they have substantial lethal as well as inhibitory properties—and so the questions arise: (1) what constitutes "a suitable bacteriostatic," and (2) what concentration is "sufficient"?

The position is further complicated by the fact that the B.P. states that the chosen substance shall also be compatible with the medicament, and it also draws attention to its possible loss by absorption into the rubber closure of the container. On these premises, therefore, the basic requirements for "a suitable bacteriostatic" may be summarised briefly under the three headings:

(1) ability to prevent the growth of, and preferably to kill, contaminating micro-organisms; (2) compatibility with the medicament, even on long storage and (3) low absorption rate into rubber; to which may be added (4) absence of toxicity to the patient in the quantities employed in the injection.

Each of these is probably equally important, but from the microbiological aspect items (1) and (3) are the most significant, and it is these which are discussed in this paper.

Experimental

Antimicrobial properties. Although five compounds are officially recommended in the B.P. as bacteriostats, phenol in 0·5 per cent concentration is the generally accepted standard against which any other suggested bacteriostat should be assessed. But this is not an easy matter, largely because phenol, as already stated, has considerable bactericidal as well as bacteriostatic properties and also because different types of compounds have quite different ranges of activities against the various groups of organisms. These points are illustrated in Tables IA and IIA, which show the relative lethal and inhibitory properties of the B.P. bacteriostats.

TABLE I

Lethal properties of bacteriostats

Bacteriostat, per cent	Lethal times (in hours) for			
	Staph. aureus	*E. coli*	*Ps. pyocyanea*	Mould spores
A. B.P. Bacteriostats				
Phenol 0·5	8–24	8	8	>24
Cresol 0·3	2–4	1	1	>24
0·15	>24	>24	>24	N.A.
Chlorbutol 0·5	8–24	1	2	>24
0·3	N.A.	>24	>24	N.A.
Chlorocresol 0·1	1	1	1	3
0·05	>24	2–4	2	N.A.
Phenylmercuric nitrate 0·001	4	1	1–4	3
B. Other Bacteriostats				
Benzyl alcohol 2	1	1	1	>24
1	6–24	24	6	N.A.
Dichlorobenzyl alcohol 0·1	1–3	1	1	>24
Cetrimide 0·01	1	1	6–24	2
Methyl paraben 0·2	8	24	24	N.A.
Propyl paraben 0·02	4	>24	N.A.	N.A.
Mixed parabens 0·2 + 0·02	4–8	1–3	8–24	>24

>24 = substantial reduction in count, but less than 99 per cent, after 24 hours.
N.A. = no substantial reduction in count after 24 hours.

In each of these assessments, tests were made with (*a*) mixed cultures of several strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas pyocyanea* grown in nutrient broth, each group being treated separately, and (*b*) aqueous suspensions of spores from various species of *Penicillium*, *Cladosporium*, *Aspergillus* and *Mucor*. The bacterial cultures comprised as many as ten strains of each type, a number of which had been supplied earlier by Mr. J. W. Lightbown of the National Institute for Medical Research, London.

The lethal tests were made with solutions in distilled water. Each solution was inoculated with the chosen test organisms to give a final concentration of $1 \times 10^6 - 1 \times 10^7$ viable cells per ml. At intervals ranging between 1 hour and 24 hours, platings were made, and the minimum times recorded at which there were no survivors in 0·01 ml. of the solutions, that is, when there was a virtual kill of over 99·99 per cent.

The inhibitory tests were made with solutions in tryptic digest broth diluted tenfold with water and with 0·1 per cent of glucose added. The same organisms were used as in the lethal tests, but the amount of inoculum was much lower, in the range of 10,000 to 50,000 viable cells per ml., and the incubation was for 5 days at 25°. Diluted broth was used instead

of normal nutrient broth so that the organic content of the solution could be kept to a reasonable minimum; it was, of course, quite adequate to support the growth of the chosen test organisms.

The results reported in Tables IA and IIA serve to show (1) the different relative activities of the five B.P. bacteriostats against the different groups of organism examined, (2) the high resistance of *Staph. aureus* in comparison with the Gram-negative types, (3) the high resistance of *Ps. pyocyanea* in relation to that of *E. coli*, and (4) the inefficacy of phenol, cresol and chlorbutol as lethal agents for mould spores. These values must not, of course, be taken as absolute; they apply only to the particular conditions of the experiments made and the cultures used—if other cultures and conditions had been employed then different values might have been

TABLE II

INHIBITORY CONCENTRATIONS OF BACTERIOSTATS

Bacteriostat	Concentration per cent inhibitory to			
	Staph. aureus	*E. coli*	*Ps. pyocyanea*	Mould spores
A. B.P. BACTERIOSTATS				
Phenol	0·2	0·2	0·16	0·05
Cresol	0·16	0·1–0·16	0·2	0·05
Chlorbutol	0·25	0·16	0·16	0·2
Chlorocresol	0·025	0·02	0·05	0·007
Phenylmercuric nitrate	0·00013	0·0005 – 0·0001	0·0002	0·00001
B. OTHER BACTERIOSTATS				
Benzyl alcohol	0·5	0·4–0·5	0·4	0·4
Dichlorobenzyl alcohol	0·025	0·04–0·05	0·04–0·05	0·016
Cetrimide	0·0004	0·001	0·04	0·0005
Methyl paraben	0·2	0·1	0·14	0·05
Propyl paraben	>0·02	>0·02	>0·02	0·015
Mixed parabens	0·1	0·1	0·1	0·025

obtained—but they illustrate clearly the points enumerated above. The Tables also indicate the narrowness of the margin, particularly with cresol, chlorocresol and chlorbutol, between the effective lethal concentration and the minimum inhibitive concentration, and this is further brought out in Table IA, which shows the profound effect of concentration on the lethal activities of these compounds, solutions at even only half their recommended strengths having considerably reduced, or sometimes practically no lethal powers.

Tables IB and IIB record the results obtained by similar tests with other bacteriostats. Of these, cetrimide and the parabens (esters of *p*-hydroxybenzoic acid) are well known, the latter often being used as mixtures in the ratios of 5 to 1 or 10 to 1 by weight; benzyl alcohol has been recommended at a concentration of 0·9 per cent or greater[1], particularly for the preservation of ophthalmic solutions[2], and 2:4-dichlorobenzyl alcohol is one of a series of derivatives of benzyl alcohol, the properties of which are described elsewhere[3]. The results again illustrate, as with the B.P. bacteriostats, the differing activities of the compounds against the various types of organisms, and also the narrowness of the margins in some cases between their effective lethal concentration and minimum inhibitory concentration.

Absorption by rubber. Attention has already been drawn to the loss by absorption of bacteriostats from injections in rubber closed containers[4], and in the same paper the partitioning ratios of the B.P. bacteriostats between rubber and water were given. By similar methods, the partitioning ratios for cetrimide, methyl and propyl parabens and 2:4-dichlorobenzyl alcohol have now been determined, and they are given in Table III; the values for the B.P. bacteriostats as found in the earlier paper[4] are also included for convenience. They show the unsuitability of

TABLE III

THE PARTITIONING RATIOS OF BACTERIOSTATS BETWEEN RUBBER AND WATER

Bacteriostat	Approximate distribution per cent between	
	Rubber	Water
Phenol	25	75
Cresol	33	67
Chlorocresol	85	15
Chlorbutol	80–90	10–20
Phenylmercuric nitrate	>95	<5
Benzyl alcohol	15	85
Dichlorobenzyl alcohol	90	10
Cetrimide	80–95	5–20
Methyl paraben	10	90
Propyl paraben	30–40	60–70

chlorocresol, chlorbutol, phenylmercuric nitrate, dichlorobenzyl alcohol and cetrimide in this context and the comparative acceptability of phenol, benzyl alcohol and methyl paraben.

DISCUSSION

Three statements are made in the B.P. about bacteriostats added to injections: (1) they must not interfere with the therapeutic efficiency of the drug or cause a turbidity, (2) they must be capable of preventing the growth of micro-organisms (in a similar context the Therapeutic Substances Regulations, 1952, use the phrase "the common contaminating organisms, both aerobic and anaerobic"), and (3) "A bacteriostatic need not be added if the medicament itself has sufficient bacteriostatic power". It also adds a warning about their absorption into rubber, but beyond these general statements it offers no guidance for the assessment of substances potentially useful as bacteriostats, neither does it indicate how bacteriostatic power can be measured. Attention has already been drawn to this[5], and in fact, various criteria have been suggested from time to time (*see*, for example, refs. 6–8).

In terms of antimicrobial activity, it is reasonable to expect that the preparation shall be effectively lethal as well as inhibitory to all types of organisms, including moulds (although it is too much to expect lethal action against the bacterial spores which have notoriously high resistances). The term "effectively lethal" is now generally accepted as implying the virtual sterilisation of a moderately heavy inoculum of suitably resistant vegetative bacteria within 24 hours, and this is, in fact, the performance level of the recognised yardstick for such assessments, namely, phenol in 0·5 per cent concentration. Such a standard has

already been put forward by the Ministry of Health Sub-Committee on Bacteriostatics[8], along with the suggestion that *Staph. aureus* and *Ps. pyocyanea* should be the organisms of choice. As representing the most resistant of the Gram-positive and Gram-negative groups of bacteria, they are admirably suitable for this purpose, but in order to obtain a satisfactory and reliable spread of resistance, several strains of each should be used, preferably of recent isolation. Even so, the values obtained cannot be taken as absolute, because one still encounters certain strains and types of organism, or particular conditions of culture, which exhibit unusually high resistance. Thus, some of the water-borne strains of *Pseudomonas* are more resistant to bacteriostats than is *Ps. pyocyanea*; likewise, although cetrimide in 0·02 per cent solution is normally effective, and a 0·01 per cent solution has been found satisfactory with procaine penicillin suspensions[8], there is a report of the growth of *Ps. pyocyanea* even in a 1 per cent solution[9].

In the opinion of the author, mould spores should always be included amongst the test organisms, because they will often grow where bacteria do not, and there is at least one finding on record of the growth of *Cladosporium* in insulin solution containing 0·17 per cent of cresol[5].

Further points for consideration are the conditions under which the bacteriostat is to be employed and their effect on its activity. In this respect, pH value, temperature and the presence of organic matter are probably the most significant. Organic matter in the form of amino acids, proteins, etc., always suppresses the activities of antimicrobial agents, but interference from this source is likely only to be small, because with a few exceptions the organic content of parenteral injections is low. The temperature effect can be variable depending on the type of substance being examined, but from general considerations of the conditions of storage and use, a test temperature of about 25° seems to be the most suitable. The effect of pH value can be more profound; most of the phenolic substances, for instance, tend to lose their activities as they move into the alkaline range. Fortunately in this respect most injections have either neutral or slightly acid reactions, and this in some cases allows the amount of added bacteriostat to be reduced. Such an example is found with insulin injections with pH values of about 3 in which as little as 0·2 per cent of phenol has been shown to be adequate, except possibly against mould spores[5].

Finally, there is the question of absorption by the rubber closure. If an injection is to contain sufficient of a bacteriostat, even after long periods of storage, it follows that the partitioning ratio of the bacteriostat between rubber and water should be low; failing this, either an adequate margin of concentration must be allowed in the initial solution, or some other precaution must be taken to conserve the concentration of the bacteriostat in the injection. In this context, suitable precautions, along with methods for assessing partitioning ratios, have already been described[4].

In conclusion, it may be said that although the five bacteriostats as recommended in the B.P. have proved satisfactory for most purposes, they have their limitations and disadvantages. In particular, they are

all readily absorbed, with the exception of phenol, into rubber, and so lost as effective agents in the preparation. There is room, therefore, for other suitable substances, and in fact many have already been proposed. Others will undoubtedly appear from time to time, but as yet there is no approved method for assessing their practical value. This is a matter needing careful consideration and controlled experimentation, and the criteria as outlined in the earlier part of this paper are put forward as a basis for discussion.

Acknowledgment. I wish to thank Mr. C. Bowler, Mr. D. V. Carter and Mr. A. Royce for the experimental data recorded in this paper.

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DISCUSSION

The paper was presented by the AUTHOR.

The CHAIRMAN. The B.P. might give further guidance by providing a testing procedure to ensure the bacteriostat was suitable.

MR. G. H. WRIGHT (Leeds). Has any research been done on polythene or other plastic closures?

DR. A. M. COOK (London). How would the Author recommend the test to be carried out, against a specific resistant strain of one organism or as a spectrum test against a number of organisms? He agreed that any test should include fungal spores.

DR. F. HARTLEY (London). The pharmacist must be satisfied that there was no danger of either mould or bacterial contamination. Tables I and II showed that the chosen concentration of bacteriostat inhibited mould spores below the lethal concentration. Unless there was a danger of pathogenic fungi, there was a wide margin of safety. Was there any point in having more than one substance?

MR. K. HOLLAND (Romford). Was it not time to stop using multi-dose containers?

MR. W. T. WING (Newcastle on Tyne). Tables I and II showed that the lethal and inhibiting effects of cetrimide were high, it was non-volatile but the partition ratio between rubber and water was high. If rubber closures were previously equilibrated with the chosen concentration of cetrimide, it might be satisfactory. Were the rubbers in Table III of different composition and did the contact time vary or were there defects in the assay?

DISCUSSION

DR. K. R. CAPPER (London). What media had been used in the tests. Did they contain inactivating substances?

The AUTHOR replied. Certain plastics were relatively non-absorbent but the majority were—some more so than rubber. There was no such thing as specific resistance amongst organisms. He had chosen staphylococci and coli and several strains of *Ps. pyocyanea.* A spectrum test was necessary, as a selective type of compound which was active against Gram-positive but not Gram-negative organisms was useless. The most commonly encountered contaminants were staphylococci and Gram-negative organisms, particularly water borne organisms growing at 25° rather than 37°. It was not unreasonable to aim for a single substance which would be effective against bacterial as well as common mould spore contamination. Moulds could gain access to a sterile room as readily as bacteria. As a bacteriologist he ought to condemn multi-dose containers, but there were other considerations. Although cetrimide was probably stable, difficulties arose because of its large absorption rate and reduced activity in presence of traces of organic matter. The lethal tests were made in aqueous solution and the media used included appropriate inactivators.

THE FACTORS INFLUENCING STERILISATION BY LOW PRESSURE STEAM

PART I. DESIGN AND INSTRUMENTATION

BY T. E. BARSON, F. PEACOCK, E. L. ROBINS AND G. R. WILKINSON

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Received June 1, 1958

A vessel has been designed for the study of the factors influencing sterilisation by steam at low pressure. Instruments have also been constructed for the conversion of pressure and the volume of condensate into electrical signals suitable for application to a recording potentiometer. Commercially available steam traps have been tested, compared, and found unsuitable and a modified steam trap has been produced, which is considered to be more efficient for this purpose than those normally used.

MOIST heat derived from steam under pressure is still considered to be the most satisfactory means of attaining sterility, and an exposure of at least 20 minutes to saturated steam at 121° C. (250° F.) is required[1].

According to Bowie[2], about 90 per cent of the sterilisers in British hospitals and pharmacies are obsolete and include representatives of all developments and stages in design since 1870, and he gives details of the conditions obtaining in such sterilisers. In 1956, Howie and Timbury[3] produced results obtained from a series of sterilisers showing the inadequacy of certain types and methods of sterilisation; these workers also pointed out that the sterilised content of an autoclave could be recontaminated during the drying process. Other factors which influence the conditions in a steam steriliser are the presence of air, which may hinder the penetration into fabrics by steam, and radiation effects from the chamber walls, and these may bring about localised unsaturation of the chamber atmosphere and possible non-sterility of the contents. It is usual to remove initial air from the chamber and its contents by one of two methods: (*a*) downward displacement, or (*b*) application of a vacuum derived from an ejector or a reciprocating pump, the former producing a vacuum of some 15 to 20 in. of mercury and the latter 28 to 29 in. For experimental work it is also possible to employ a high vacuum pump, so that the residual air pressure is only about a few millimetres of mercury. Evacuation[4] and downward displacement[2,5], have their protagonists, but the efficiency of sterilisation is dependent in addition upon factors such as the packing of the dressings and loading of the steriliser.

Metal drums, particularly if they are overpacked, are difficult to sterilise, as reported, for example, by Howie and Timbury[3], and penetration of steam is thought to be better into fabric wrapped packs.

The purpose of this investigation was to find the optimum conditions for sterilisation, and to study the construction of a suitable steriliser in which to bring this about. In the experiments we describe, steam is raised in a separate boiler and used as the sole source of heat.

Equipment and Installation

An experimental steriliser, 16 in. diameter by 36 in. long internal dimensions, with a steam jacket lagged externally, was constructed and provision made in the chamber wall for alternative steam inlets and condensate outlets at various points. The position and form of the condensate outlet and steam trap could also be varied and entries for thermocouple wires were provided.

To simulate conditions obtaining where sterilisers are commonly in use, provision was made for reducing the main steam pressure (80 lb. per sq. in.)

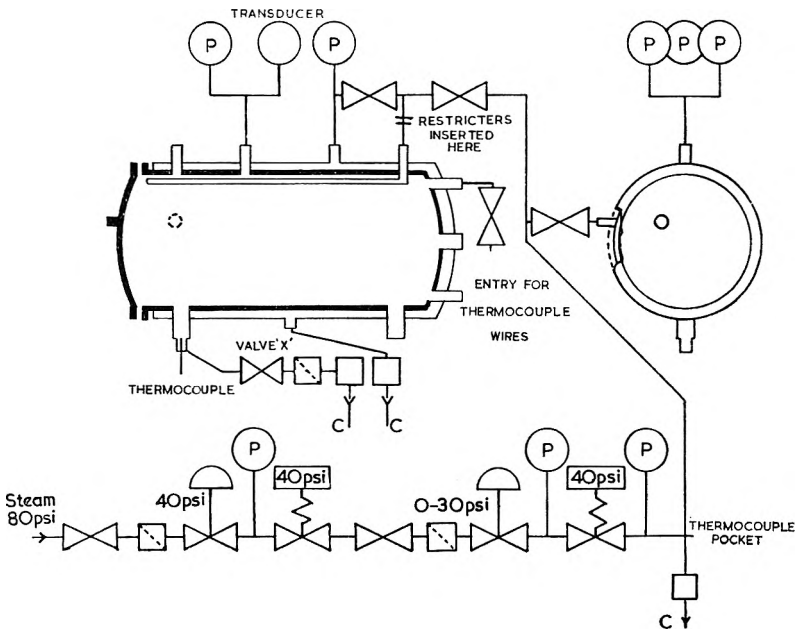


FIG. 1. General arrangement of steam supply and condensate flows connected with the steriliser. Graphical symbols B.S. 1553.

to working pressure. This system and the other connections are indicated in Figure 1. The condensate from the chamber and jacket were taken separately to waste. Only the jacket of the steriliser and the steam pipe to the first reducing valve were lagged, as is common practice in installations of this type.

A Bristol's Dynamaster Recording Potentiometer with integral cold junction compensation, modified so that it could also be used at will as an indicator on any of its six channels, was used for all temperature readings, and also by adaptation for recording pressures and volume of condensate produced by the chamber steam trap. The thermocouples were of iron: constantan wire sheathed in woven glass fibre and coated with Araldite resin as a further protection. They were inserted in the chamber through modified Kontite fittings, with rubber gaskets to ensure steam and water tightness.

The commercial instruments measuring changes in pressure within the chamber proved insensitive and irregular in operation and it was necessary to construct one to meet our own needs (Fig. 2). A transducer was connected in a Wheatstone's bridge circuit[6] and this device gave a suitable signal for application to the potentiometer, the effect of the automatic cold junction compensation being offset by introducing into the circuit an iron:constantan thermocouple immersed in melting ice. No other compensation for change in ambient temperature was found necessary, except to surround the bridge network by a box to prevent draughts impinging on the resistors.

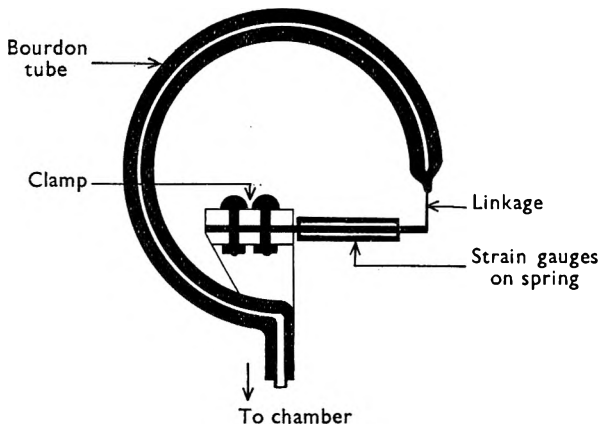


FIG. 2. Transducer. Strain gauges were of etched foil (Ferry) and were cemented on the spring with araldite strain gauge cement.

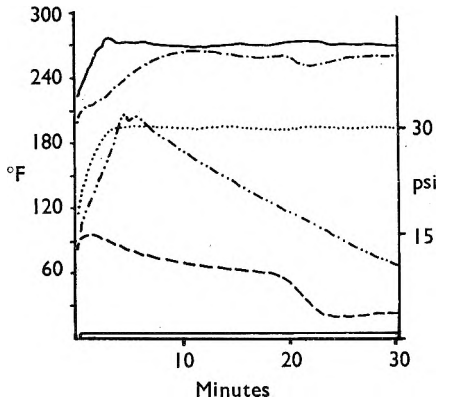
To measure the amount of steam used, and the time of its use, a tilting bucket mechanism was devised. Each time the bucket emptied an electrical impulse recorder made a mark on the chart which also recorded temperatures and pressure.

CHECKING OF INSTRUMENTS

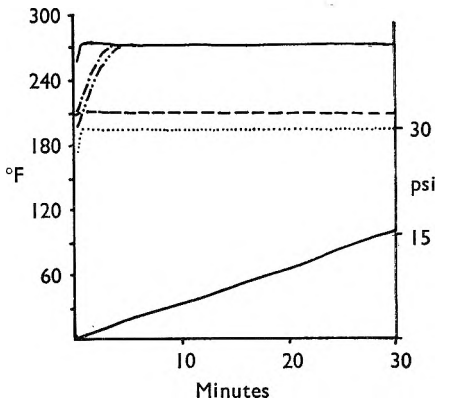
Thermocouples and recorder. The thermocouples were calibrated against melting ice 0° C. (32° F.) and boiling distilled water 100° C. (212° F.) with due correction for atmospheric pressure and using N.P.L. standardised mercury-in-glass thermometers for day-to-day checks. Adjustments for calibration errors in the recorder were according to the makers' instructions.

The effect of the water equivalent on the response of the thermocouples was checked by first allowing them to equilibrate with ambient temperature (about 65° F.) and timing the period required for a 75° C. (135° F.) change in temperature. The time was five seconds and this was considered an adequately rapid response.

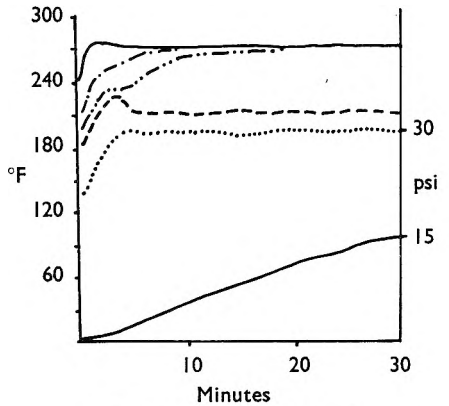
To estimate the effect of conduction of heat along the thermocouple wires one was heated some 12 in. from the junction and it was some minutes before any significant increase in temperature was indicated.



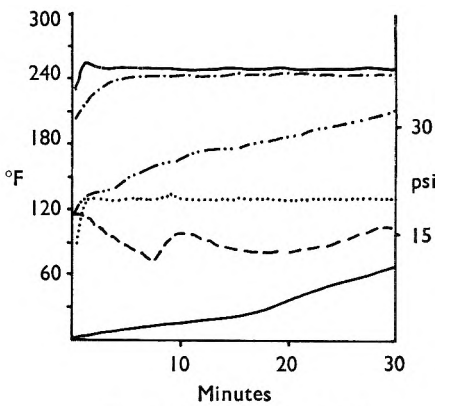
A. Experiments Nos. 1 and 11.



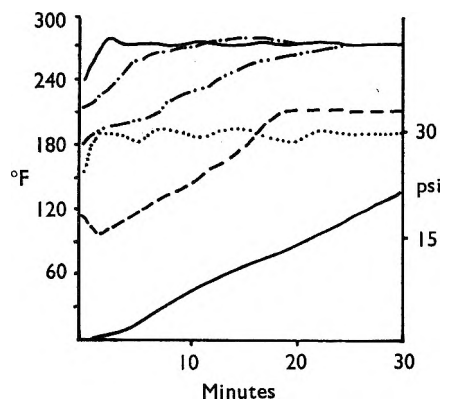
B. Experiments Nos. 2, 12, 14, 16.



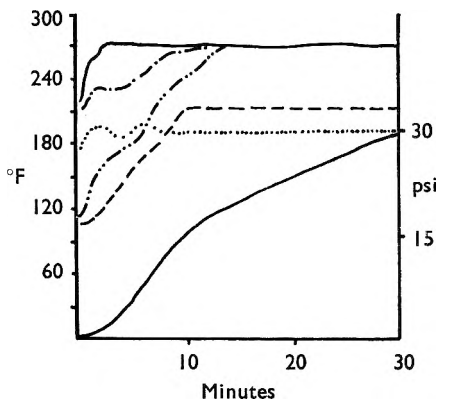
C. Experiment Nos. 3–8.



D. Experiment Nos. 9, 10.



E. Experiment No. 13.



F. Experiment No. 15.

FIG. 3. Operating conditions shown by temperatures, pressure and steam usage. For explanation see text. Key on facing page.

The effect of radiation from the inner surfaces of the chamber was examined by fitting the junctions with thin aluminium shields which allowed free circulation of vapour but prevented the absorption of radiation. It was found that their behaviour was identical with unscreened junctions similarly positioned in the chamber.

Pressure transducer and gauges. These were checked against a mercury manometer in an upward and downward direction.

Tilting bucket. The volume per delivery of each bucket was measured over a period and found to be sufficiently consistent for comparative purposes.

Experimental

Arrangement of inlet and outlet. The first experiments were made by introducing the steam through a sparger lying horizontally along the top of the chamber and collecting the condensate in a single large outlet hole at the bottom front of the chamber connected to a steam trap.

Using downward displacement of air, a thermostatically controlled steam trap, which operated in the near-to-steam range, and an empty chamber, tests were made at varying steam pressures. At the same time the disposition of the thermocouples within the chamber was examined for variations in temperature when the chamber was in equilibrium.

No significant difference between the temperatures of the thermocouples wherever disposed was observed once equilibrium had been achieved, but when they were positioned at different levels, including in the outlet drain, they reached the operating temperature successively from top to bottom, the one in the drain being the last to achieve the maximum temperature. This phenomenon was most noticeable when the chamber was heated up from cold with no steam-heat applied to the jacket.

Superheat of the steam was checked during each experiment from the thermocouple and pressure gauge inserted into the supply pipe and reference to steam tables. At no time did the steam temperature differ by more than 1° F. from the temperature obtained by referring the indicated pressure to the tables. A satisfactory determination of the quality of the steam by the bucket calorimeter was found to be impracticable.

Variations in steam inlet. When the rate of entry of steam at 30 lb. per sq. in. into the chamber was varied by throttling with restricters in the form of annuloid discs, changes were seen. The time to attain equilibrium was the same for $\frac{1}{2}$ in. and $\frac{3}{8}$ inch bore; for $\frac{1}{4}$ in. the time was prolonged and with $\frac{1}{8}$ in. much prolonged. The same thing was seen at 20 and 10 lb. per sq. in.

Effect of steam trap. During this work it was noted that the temperatures within the chamber and the steriliser drain lagged behind steam

Key to Figure 3.

——————— Temperature at the top of the chamber.
— • — • — Temperature at the bottom of the chamber.
. . . . . Chamber pressure.
— — — — Temperature in the steam trap delivery pipe.
— • • — • • Temperature in drain.
——————— Lower trace: steam usage.

temperature, and it was observed that once the trap had shut for the first time it ceased to pass air, thus causing a build-up in the air concentration within the chamber.

Since no published work on the subject of steam traps used on steam sterilisers could be traced, experiments were devised to examine this phenomenon and to investigate the behaviour of representative types of commonly used traps. Observations were made from thermocouples in the following positions: 2 in. below the top of the chamber, 2 in. above the bottom of the chamber, in the drain leading from the chamber to the steam trap, and in the steam trap condensate outlet immediately below the trap.

Experimental conditions. Throughout these experiments reproducibility could be established only if the following conditions were observed. The autoclave chamber remained empty. The jacket remained heated during the intervals when the chamber was opened. The chamber, initially at operating conditions, was emptied of steam and the door opened to the atmosphere for a period of ten minutes. The interval was kept as short as possible between closing the door and admitting steam to the chamber as it was found that initial superheating of the admitted steam was affected by the length of this interval.

Each steam trap was installed in the manufacturer's recommended position, except in Experiment No. 11 where this alteration was deliberate and suggested as a possible way of ensuring the venting of air, as the bellows would then be constantly immersed in condensate.

The experiments with the various steam traps have produced the following information, which should be read in conjunction with the numbered diagrams of examples of the records obtained (Fig. 3).

Experiment No.	*Conditions of Test*	*Results*
1.	All outlets from the chamber closed (Fig. 3A).	Uniform conditions in the chamber were not reached, and the temperatures, particularly that in the drain, dropped slowly due to the accumulation of air and of condensate. Layering was very pronounced. Little steam was used within the chamber.
2.	A slightly opened valve in place of the trap so that steam was being blown off at a moderate rate during the whole of the cycle (Fig. 3B).	Uniform conditions were reached rapidly and smoothly, and the temperature of the drain soon attained steam temperature.
3.	Steam trap with liquid-filled bellows operating in the near-to-steam range (Fig. 3C). (See also Fig. 23, cf. ref. 7.)	A steady attainment of steam temperature throughout the chamber and drain. The peak, as recorded in the steam trap delivery,

		showed a time lag in the operation of the valve, probably due to the thermal capacity of the liquid-filled bellows. Slight initial superheating took place in the top of the chamber.
4.	Liquid-filled bellows operated; low pressure type (Fig. 3C). (See also Fig. 23 of ref. 7.)	Another steady, but slightly more rapid attainment of steam temperature. Some slight accumulation and subsequent release of air inside the drain was indicated by variations in the temperature in the drain.
5.	Liquid-filled bellows operated (Fig. 3C). (See also Fig. 3 of ref. 7.)	Again steady concurrence of chamber temperatures, but the trap appeared to allow greater accumulation of air in the chamber drain before release. The temperature in the drain was not completely stable.
6.	One float-operated valve and one liquid-filled bellows operated valve incorporated in the same body but operating independently (Fig. 3C). (See also Fig. 19 of ref. No. 7.)	The initial rise in the temperature at the bottom of the chamber and in the drain was not so great as in Experiment Nos. 3, 4 and 5. The eventual coincidence of the three temperatures was reached sooner, and no subsequent variations in the drain temperature occurred.
7.	A single valve controlled by a combined float and bi-metallic strip mechanism (Fig. 3C.)	The temperature characteristics of the chamber thermocouples were closely similar to Experiment No. 6, but fluctuations in the drain temperature indicated less effective elimination of air.
8.	A pressure - compensated balanced valve operated by a bi-metallic strip mechanism, with provision for adjusting the operating temperature. (See Fig. 21 of ref. 7.)	Steady attainment of steam temperature. The valve in the trap was found to stick frequently, making the operating temperature adjustment difficult to set and operation inconsistent. (This appeared to be a characteristic of the valve design.) Again the record of this experiment was closely similar to that of Experiment No. 6 and that used in Figure 3 is illustrative of the type of trace obtained.

9 & 10.	Float-operated valve; working pressure 20 lb. per sq. in. (Types I and II) (Fig. 3D). (See also Fig. 20 of ref 7.)	Uniform temperature inside the chamber was never attained throughout the duration of the experiments; the drain temperature remained very low, and the flow of condensate recorded was much less than with any other trap, so indicating a slow rate of heating.
11.	The same trap as in Experiment No. 1 but installed in an inverted position (Fig. 3A).	No improvement was obtained by inverting the trap.

The differences in the chamber and drain temperatures appear to be explained by the layering of air and steam within the chamber to varying degrees, that is, the separation into layers of increasing air content downwards. It was also obvious that the air was not being satisfactorily eliminated by any of the traps tested.

The trap which appeared to perform most satisfactorily (Experiment No. 6), was now modified by including a small continuous by-pass. This allowed a test to be made which approximated to the Conditions of Test in Experiment No. 2 without large volumes of steam being wasted. The liquid-filled bellows unit, which normally operated to close the thermostatic valve, was removed. The remaining orifice was used as a by-pass, in two ways, full bore and also restricted by pieces of wire. Records 12 and 13 were obtained with orifices 2·0 and 0·65 sq. mm., respectively in area, with the jacket heated, and 14 and 15 with the same orifices, with the jacket unheated. The cold jacket made the differences more obvious.

Experiment No.	*Conditions of Test*	*Results*
12.	Trap as in Experiment No. 6, modified so as to provide a small permanent by-pass. See experimental detail. 2 sq. mm. by-pass.	Very close agreement in behaviour with Experiment No. 2. Air and condensate were rapidly eliminated, although there was a slight degree of super-heating initially at the top of the chamber; steam temperature was fairly rapidly attained and maintained in all parts, including the drain. The bucket section of the trap appeared to be working normally.
13.	As Experiment No. 12, but with 0·65 sq. mm. by-pass. (Fig. 3C.)	Less rapid elimination of air and layering was clearly indicated.

14.	As Experiment No. 12, but with 2·0 sq. mm. by-pass. (Fig. 3F.)	Similar to Experiment No. 12.
15.	As Experiment No. 12, but with 0·65 sq. mm. by-pass.	Layering suggested, with less rapid elimination of air; the complete elimination was sharply indicated by a sudden rapid rise in the delivery pipe and drain temperatures.

In an attempt to improve matters further, the same trap was again modified. The bellows were replaced and a small permanent by-pass, 0·5 sq. mm. in area, drilled in the casting in addition to the float-operated valve. Under these conditions the thermostatic bellows, float-operated valves and by-pass were all operative.

The jacket of the steriliser was heated.

Experiment No.	*Conditions of Test*	*Results*
16.	Completely modified steam trap. See experimental detail. (Fig. 3B.)	Closely similar to Experiment No. 2.

The behaviour of the modified trap produced the conditions required for the satisfactory operation of the steriliser under conditions of air elimination by downward displacement.

Discussion

It seems that commercially available steam traps are not capable of passing the volume of air met within a steam steriliser in its normal cycle of use. This defect from the point of view of our needs is a feature of their design, since thermostatic traps will still respond to hot air or steam and air mixtures and yet remain closed, although there is air on the input side. Bucket traps are equally unsuitable since they have only a small hole to vent the air in any case. A way of overcoming this shortcoming has been found and an improvement in the steam trap demonstrated.

The steriliser and instrumentation have been carefully checked and shown to be sound apparatus for further experimentation.

We wish to thank F. C. J. Caliendi of the De Havilland Aircraft Co. Ltd., Hatfield, Herts, for information concerning the strain gauges.

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THE FACTORS INFLUENCING STERILISATION BY LOW PRESSURE STEAM

PART II. THE INFLUENCE OF WATER CONTENT OF COTTON GOWNS ON EQUILIBRIUM TIMES

BY T. E. BARSON, F. PEACOCK, E. L. ROBINS AND G. R. WILKINSON

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Variations in the times to reach steam temperature by surgeons' gowns within a steam steriliser with air elimination by downward displacement and evacuation have been examined. Using surgeons' gowns equilibrated with normal atmospheric conditions, about 60 to 70 per cent relative humidity, the times taken for the gowns to attain steam temperature were variable, whichever method of air removal was employed. Gowns dried at 50° rapidly attained steam temperature and then superheated to a considerable degree. The superheat was maintained for long periods.

WE have described elsewhere[1] the design of a low pressure steam steriliser and its associated recording instruments. The same experiment uncovered the unsuitable nature of existing steam traps and led to the design of a satisfactory modified trap[1].

Using the steriliser and instrumentation outlined in the foregoing paper[1], experiments were now made to examine further the conditions within the chamber, only this time the chamber was filled.

EXPERIMENTAL

With the steam trap used in Experiment No. 3[1] a dressing drum 13 in. in diameter and 9 in. deep was packed with five folded surgeons' gowns and positioned on its edge centrally in the chamber with the folds in the gowns in the vertical plane. One thermocouple was positioned at the centre of the gowns and a second 1 in. from the outer edge. Others were in the chamber drain and steam supply. A fairly rapid attainment of temperature was recorded by the outer thermocouple but the inner thermocouple lagged considerably.

When the steam trap was replaced by the new modified steam trap used in Experiment No. 16[1] and the experiment repeated the time to attain steam temperature was much reduced, but variations occurred in the times to reach steam temperature and in the case of the longer periods slight superheat was shown by the thermocouples within the gown. The same five gowns were used for each experiment being dried successive in a hot air oven on each occasion and allowed to equilibrate in the atmosphere at a 60 to 70 per cent relative humidity to within 2 per cent of a standard weight. Examples of these variations which are too large to be accounted for by experimental error are as follows.

Time to reach Steam Temperature in minutes

Drain	*Centre of Gowns*	*Edge of Gowns*
7	23	4·5
7·5	35	7·5
4·5	24	29
4·5	More than 60	More than 60

The experiment was now repeated with the gowns dried for 24 hours in a hot air oven at 50° C., during which time they lost about 5 per cent in weight. They were then submitted to the sterilising cycle (Fig. 1). The dressings attained steam temperature almost as rapidly as an empty chamber and superheated to 6° C. (11° F.) above steam temperature. The experiment was repeated and continued until equilibrium was attained (Fig. 3). It took 59 hours for the centre of the gowns to fall to steam temperature.

Using the same arrangement of steam inlet, condensate outlet and steam trap, a vacuum pump was now attached to the lower union at the back end of the steriliser and a mercury manometer to the central one.

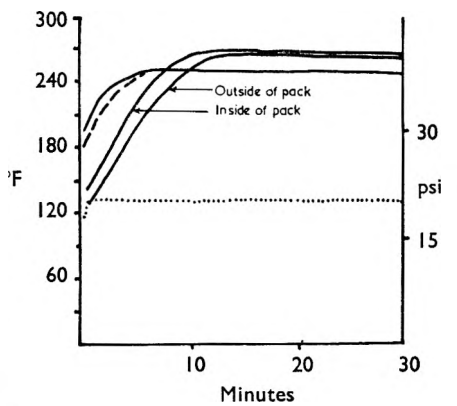

FIG. 1. Showing superheating of interior of gowns.
——— Steam pressure
– – – Drain temperature
..... Pressure

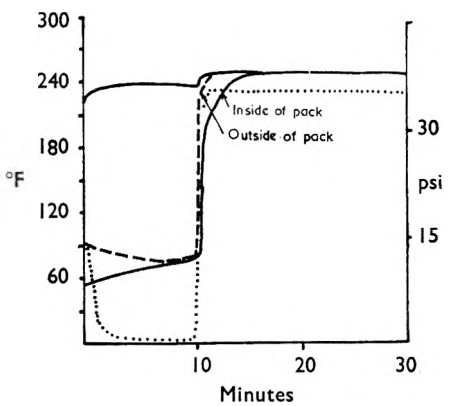

FIG. 2. Temperature of gowns attained after initial evacuation of the sterilizer.

The required degree of vacuum was pumped after first closing valve X, Figure 1 of the previous paper, on the condensate outlet, steam turned on and this valve opened as soon as the pressure within the chamber just exceeded atmospheric pressure. Evacuating to 20 in. of mercury vacuum experiments were carried out using both the unmodified and the modified steam traps and with the dressing drums filled with equilibrated gowns. The modified steam trap again behaved a little better, and steam temperature was attained in about 20 minutes. The type of trace obtained is illustrated in Figure 2. No superheating occurred.

Experiments were then made with the chamber evacuated to 40 mm. of mercury, the highest vacuum which could be achieved with a heated jacket. Steam temperature was attained in about 15 minutes and there was no superheating. The type of trace is illustrated in Figure 2.

Using dried gowns the chamber could be evacuated to 20 mm. of mercury and these conditions were used for further experiments. Steam temperature was attained in 3 minutes and the centre of the gowns superheated to 8 to 12° C. (17 to 21° F.) above steam temperature.

Discussion

The conditions within the steriliser can be modified both by external influences, and also by the physical state of the material undergoing sterilisation.

The cotton fabric used in our experiments readily attains steam temperature when air is eliminated by downward displacement if the steam trap is efficient, but the drain temperature is not found to be a reliable indication of the temperature of the steriliser contents.

In addition, superheating occurred only when the gowns had been dried to 50° C. and the period this superheat is maintained is protracted.

Rapid attainment of steam temperature and superheating which occurred only with gowns that had been dried in a hot air oven leads to a consideration of the degree of hydration of the fabric prior to autoclaving. This single factor appears to have an important effect on sterilising times and conditions.

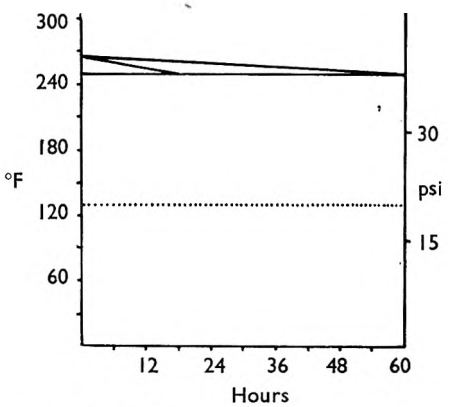

Fig. 3. Period of maintenance of superheat.

Two possible explanations suggest themselves. The first is the great difference between the thermal conductivity of dry air and air containing water vapour, and the second is that heat is evolved when the cellulose fibres re-hydrate after drying. Steam condensing on the cotton, would tend to be adsorbed to the cotton fibres, liberating its heat of hydration and thus raising the temperature locally, so causing superheating. Since cotton or cellulosic materials also possess high thermal insulation values, the conditions would be unlikely to reach equilibrium quickly with the steam external to the dressings.

This theory is supported by approximate calculations, which show that the degree of superheat in practise is close to the figure estimated by reference to tables[2] giving the heats of hydration of cellulose. Knox and Penikett[3] have reported a significant decrease in the time required to reach 115° C. (240° F.) if the vacuum obtained is below 20 mm. With the particular apparatus available and an efficient vacuum pump capable of producing a vacuum of 0·1 mm. of mercury on a closed circuit, with a capacity of 450 l. per minute, we have been unable to reduce the pressure within the chamber below 20 mm. of mercury when the jacket was heated, although the vacuum in the pipeline attached to the pump was below 5 mm. of mercury. Even under these conditions there was no great increase in the rate of attainment of sterilising temperature within the steriliser contents compared with the same chamber emptied of air by downward displacement.

Since the work reported in the paper has raised a number of contentious points it is clear that further work must be done before any tests are

designed using bacterial spores to ascertain whether ideal theoretical conditions do in fact bring about sterility.

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DISCUSSION

The papers were presented by MR. G. R. WILKINSON.

MR. D. W. HUDSON (Hove). The question of time and temperature should be considered in parallel with the destructive effect upon the fabric itself.

DR. R. M. SAVAGE (Barnet). Drums were being discarded in favour of fabric packs. The rate at which steam penetrated ordinary paper was surprising and a paper wrapped article would sterilise almost as quickly as an unwrapped one. He did not agree that there was no information about traps in the literature. Conrad in Berlin was working on the subject in 1924. Dressings containing 8 per cent or more of water might be sterilised by dry-heating, but if below this critical value, water must be added.

MR. G. SYKES (Nottingham). Sterilisers sometimes needed modification and were often mishandled. To be effective, evacuation must be to less than 20 mm. Hg. He has been told that Spirax traps were satisfactory.

MR. W. T. WING (Newcastle on Tyne). A new type of steriliser from Germany untilises a vacuum pump; this allows almost complete removal of air and penetration of steam.

MR. T. D. WHITTET (London). Sterilisation procedures were unsatisfactory in many hospitals. Some errors could be avoided by the use of temperature recording apparatus. In Sweden a nylon film was being used satisfactorily as a dressings container.

MR. WILKINSON replied. To obtain satisfactory sterilisation, the dressings must be in saturated steam, but pockets of superheat might exist where sterilisation may not take place. A balance must be achieved between sterilisation and destruction of the dressings. Drums were more easily handled than fabric packs; paper wrapping was effective unless it was damaged. He had referred specifically to the literature dealing with steam traps for sterilisers. A modified type of Spirax trap was successful. There was much evidence that removal of air was advantageous but it was difficult to achieve this without drying the dressing. He was investigating a prototype steriliser with a mechanical pump. Time and temperature were both important but what parameter was to be used? The customary thermometer at the top of the chamber or in the chamber drain did not necessarily give the true temperature at the centre of the dressings.

THE COLORIMETRIC DETERMINATION OF MORPHINE IN GALENICAL PREPARATIONS

By C. A. Johnson and Cecilia J. Lloyd

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Methods by which morphine may be determined colorimetrically are discussed and the present official method criticised. The reaction described by Pride and Stern, which depends upon the oxidation of morphine with iodic acid followed by treatment with ammonium bicarbonate and nickel chloride to give a green colour has been found satisfactory. Improved extraction procedures for the separation of morphine from extraneous material in a number of galenical preparations are suggested and application of the colorimetric reaction to morphine residues so obtained, is described.

In an investigation to find better assay procedures for galenical preparations of opium than have been used hitherto the reaction of morphine with fluorodinitrobenzene was studied in detail[1]. The procedure is applicable to preparations containing 0·1 per cent or more of morphine but not less since the size of sample required is then too large. Many colorimetric reactions have been proposed; these have been reinvestigated in a search for one which would be specific, reliable and applicable to pharmaceutical preparations containing small quantities of opium.

The nitrosomorphine reaction[2] is the official procedure in this country. Besides being a general phenolic reaction[3] the colour produced depends on many variable factors, while the presence of coloured extractive from other ingredients often means that the final colours are of different tints so that a compensating technique may be required[4]. Many of the variables were described by Stephens[5] but two pharmacopoeias have been issued since without modification of the standard method. Our investigation of the nitrosomorphine method confirmed and extended that of Stephens. We concluded that improvements could not be expected by modifying the official method.

In 1906 Georges and Gascard[6] described a determination of morphine based on the action of iodic acid followed by ammonia to give a brown colour and subsequently other workers modified the method and extended its use[7–9]. Although many reducing substances undergo this reaction morphine is one of the few alkaloids to do so. In 1946 Guarino[10] described a series of reactions claimed to provide a specific test for morphine. This depended on the formation of a violet-red colour when the oxidation product resulting from the action of iodic acid followed by ammonium carbonate was treated with ferric chloride solution. The procedure was adapted to the quantitative determination of morphine[11–13] and its specificity was evaluated by Javicoli[14]. Cramer and Voerman[15] found difficulties with Guarino's method and proposed the substitution of ferric chloride by nickel sulphate. In 1954 Pride and Stern[16] investigated the procedure and described its application to opium and to poppy capsules.

Little attention[17] seems to have been paid to Pride and Stern's work, yet it seemed to the present authors that the procedure would be satisfactory for general application to galenical preparations, provided that suitable extracts could be prepared. Much of the present work has been directed to this problem of the effective separation of morphine from interfering substances.

Examination of Experimental Conditions and Reproducibility of the Pride and Stern Reaction

As well as confirming the results of Pride and Stern we have extended their work to cover the following points.

The reproducibility of the method was checked over the range of 0·4 to 4·0 mg. anhydrous morphine on different days under similar conditions of light intensity and temperature. Beer's Law is obeyed over this range and consistently reproducible results were obtained. Complexes were developed in the dark, in daylight and in artificial light. Similar extinction values were obtained showing the reaction to be independent of light intensity.

The optical density was recorded at intervals of 15 minutes after the addition of the ammonium bicarbonate–nickel chloride reagent. Maximum absorption was developed at 90 minutes after the addition of the complexing solution and the complex was stable for at least a further 2½ hours.

Pride and Stern made all measurements within the limits 20° ± 1·5°. Since the working temperatures might be outside this range, colours were formed at temperatures ranging from 15 to 25° but without significant differences in optical density.

Pride and Stern's investigations into the effect of varying the concentration of ammonium bicarbonate in the complexing reagent were extended. Variations of ± 7·5 per cent were tolerable.

Specificity of the Method

Pride and Stern investigated the specificity of the reaction by examining firstly some of the naturally occurring alkaloids of opium and secondly a series of natural and synthetic bases, with structures closely related to morphine.

Non-phenolic alkaloids. The reported non-interference by the principal non-phenolic alkaloids occurring in opium was confirmed.

In opium, 0·1 to 0·5 per cent of an "unknown base" has been quantitatively determined by the United Nations Secretariat for Opium Analysis[18]. Isolation of this base, believed to be hydrocotarnine, a hydrolysis product of narcotine, was made from a sample of Turkish opium and a suitable portion taken for colour development. Although visually there was no apparent reaction with either iodic acid or ammonium bicarbonate–nickel chloride reagent an absorption curve of extinction against wavelength between 400 and 800 mμ gave a calculated E(1 per cent, 1 cm.) at 670 mμ of 4·4. (The E(1 per cent, 1 cm.) for morphine at 670 mμ was 54·2.) On the assumption that the unknown base occurs in opium to a maximum

extent of 0·5 per cent the amount of interference would appear to be negligible. Recoveries of morphine determined in the presence of amounts of the unknown base in excess of what would occur naturally were quantitative.

Phenolic alkaloids. Of these only pseudomorphine is mentioned by Pride and Stern. Although this alkaloid was obtained by Pelletier it is still not known whether it actually occurs in opium or is formed during extraction[19]; the amounts obtained are small, about 0·02 per cent. The "minor phenolic alkaloids" are known to occur, possibly up to 2 per cent.

Pseudomorphine. A sample of pseudomorphine was prepared by the method of Polstorff[20].

The liberation of iodine from iodic acid by pseudomorphine and the formation of a brown colour which merely deepens on the addition of the ammonium bicarbonate–nickel chloride reagent was confirmed. The absorption curve of the complex shows minimum absorption at wavelengths >650 and the calculated E(1 per cent, 1 cm.) at 670 mμ was 1·9. The amount of interference when the method is applied to natural products is therefore negligible and a correction procedure detailed by Pride and Stern is unnecessary. Recoveries of morphine in the presence of amounts of pseudomorphine in excess of that which would be obtained from natural sources were quantitative.

Minor phenolic alkaloids. These are porphyroxine-meconidine, the substance responsible for the formation of the red-coloured material on treatment of opium extracts with acid, representing about 0·5 per cent of the opium, and three alkaloids, laudanine and the related codamine and narcotoline, together representing about 1 per cent of the opium. No supply of these alkaloids was available and they were therefore extracted from a sample of opium by the method of the United Nations Unified Analysis of Opium.

Porphyroxine-meconidine. The fact that iodine was liberated from iodic acid could not readily be seen owing to the dark red colour of the solution but was verified by shaking with carbon tetrachloride which became purple after about 2 minutes. Addition of the ammonium bicarbonate–nickel chloride reagent produced a yellowish-brown solution which on standing gave a dark brown turbidity. After removal of the precipitate by centrifuging the extinction was negligible.

Recoveries of morphine in the presence of such proportions of porphyroxine–meconidine as would be extracted with it from an opium preparation, however, were quantitative; the reaction mixture failed to show the formation of a precipitate during the time that the green morphine complex was stable.

Laudanine, codamine and narcotoline. These three alkaloids are collectively extracted by the United Nations method, and were not separated before applying the colour reaction.

Iodine was liberated from the iodic acid; addition of ammonium bicarbonate–nickel chloride gave a yellow-brown solution which on standing produced a greyish-green turbidity. At greater dilutions the reactivity of these alkaloids appears to be so much reduced that no

precipitate is formed while the morphine complex is stable. Recoveries of morphine in the presence of these phenolic alkaloids were quantitative.

Application to the Determination of Morphine in Galenical Preparations

The United Nations Unified assay of opium for alkaloids recommends a 3 : 1 chloroform-*iso*butyl alcohol mixture for the extraction of morphine. We have found this to be satisfactory for the preparations discussed below.

Preparation of Standard Samples of the Galenicals under Test

Since many official preparations containing morphine use Camphorated Tincture of Opium which is made from Tincture of Opium, the latter was taken as a standard, samples being prepared from both Turkish and

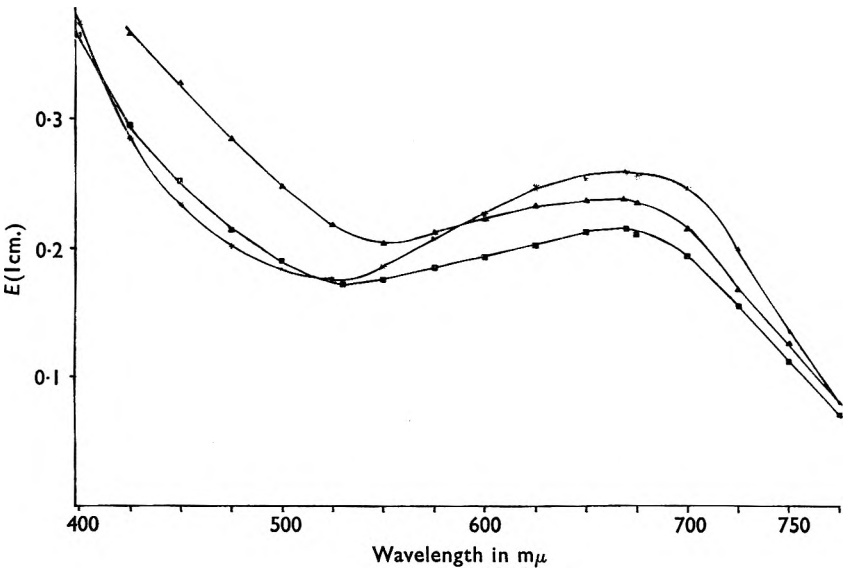

Fig. 1. Absorption curves of colours developed from morphine standard and that obtained from Camphorated Tinctures of Opium B.P.
—*—*— Morphine standard.
–▲–▲– Camphorated Tincture of Opium B.P. prepared from Indian opium.
–■——■– Camphorated Tincture of Opium B.P. prepared from Turkish opium.

Indian opiums. The anhydrous morphine content of the prepared tinctures was determined by the 2:4-dinitrofluorobenzene precipitation method[1].

Camphorated Tincture of Opium B.P. A direct extraction of the morphine from ammoniacal solution with chloroform-*iso*butyl alcohol mixtures led to emulsification. Removal of the alcohol on the steam bath and addition of 0·05N HCl to the residue gave a solution which was not only strongly coloured but had a pH above the critical value of 1·6 established by Pride and Stern[16].

A procedure similar to that recommended for the preliminary separation of morphine from extraneous material in galenicals was adopted[1].

The preparation is evaporated to a small volume and after liberation of the free base mixed with aluminium oxide to form a column. The alkaloid is then eluted from the column with a solvent. This method has the advantage of retaining much extraneous material, avoiding the formation of emulsions and requiring a minimum of solvent for extraction. Satisfactory results were obtained with this method. pH measurements on the solution before colour development ranged from 1·48 to 1·52, and of the complex they varied between 7·98 and 8·03. Pride and Stern have shown that the pH of the solution after colour development should be 8·0 $\pm$ 0·05. Blank mixtures containing oil of anise, benzoic acid and camphor gave no colour by the same procedure. Absorption curves for the morphine complexes derived from Turkish and Indian opiums and pure morphine are shown in Figure 1.

The recommended method of assay is as follows.

Apparatus. Unless otherwise stated all optical densities were recorded on a Unicam S.P. 600 spectrophotometer using 1 cm. cells.

Reagents. Dry ammonium bicarbonate. Ammonium bicarbonate B.P. 1953 dried over silica gel, at room temperature, and stored in the same manner. Chloroform—*iso*butyl alcohol mixture. Chloroform B.P. 3 parts, *iso*butyl alcohol 1 part. Iodic acid solution. A 4·5 per cent w/v aqueous solution of reagent grade iodic acid. Ammonium bicarbonate–nickel chloride reagent. Transfer 8 g. of dry ammonium bicarbonate to a 100 ml. graduated flask, add 25 ml. of 4M ammonium chloride solution, 20 ml. of N ammonia solution and 10 ml. of a 1 per cent w/v aqueous solution of nickel chloride hexahydrate and dilute to about 90 ml. with water. Stopper the flask, shake vigorously until the ammonium bicarbonate has dissolved and dilute to volume with water. This reagent should be freshly prepared but the various solutions from which it is made, with the exception of the N ammonia, may be kept as stock solutions.

Method

Take 10 ml. of sample in a small dish and evaporate to dryness on a steam bath. Triturate the residue with 1 ml. of dilute solution of ammonia. Add aluminium oxide gradually and continue triturating until a dry free-flowing powder is obtained. Transfer the powder to a dry chromatographic tube about 40 cm. in length and 1·5 cm. in diameter, previously plugged lightly above the tap with cotton-wool. Remove any adhering powder from dish and pestle with cotton-wool moistened with *iso*butyl alcohol and add to the tube. Insert the lower end of the tube through a bung fitting into the neck of a 150 ml. separator and elute with 50 ml. of chloroform-*iso*butyl alcohol mixture, adjusting the rate of elution to about 1·5 ml. per minute. Wash the solution in the separator with 10 ml. of water. Allow to separate. Filter the organic phase through a cotton-wool plug moistened with solvent into a 150 ml. beaker. Shake the aqueous phase with 10 ml. chloroform-*iso*butyl alcohol and after allowing to separate, filter the organic phase into the same beaker. Wash the filter with a little more of the solvent mixture and evaporate the solvent on a steam bath under a gentle current of air. Heat the residue on the

steam bath for a further 5 minutes to ensure removal of ammonia. Take up the residue in 5 ml. 0·5N hydrochloric acid accurately measured, gently warming for a few seconds on a steam bath if necessary. Filter the solution through a Whatman No. 41 filter paper into a 50 ml. graduated flask washing the beaker well with small quantities of water and transferring to the filter. Cool to laboratory temperature if necessary, then make up to volume with water. Take 10 ml. of the solution prepared above in each of two 25 ml. graduated flasks. To one (the experiment) add 2 ml. of the iodic acid solution with thorough mixing and after exactly

TABLE I

COMPARISON OF THE DETERMINATION OF MORPHINE BY THE B.P. METHOD AND THAT OF PRIDE AND STERN IN FIVE SAMPLES OF CAMPHORATED TINCTURE OF OPIUM B.P.

	Per cent w/v anhydrous morphine		
	Calculated	B.P. method	Proposed method
Sample A (Indian)	0·057	0·060 0·060	0·057 (Mean of 16 results; range 0·056 to 0·059)
Sample B (Indian)	0·050	0·053 0·054	0·051 (Mean of 12 results; range 0·049 to 0·052)
Sample C (Indian)	0·048	0·051 0·050	0·048 (Mean of 8 results; range 0·047 to 0·049)
Sample D (Turkish)	0·053	0·058 0·057	0·054 (Mean of 8 results; range 0·053 to 0·055)
Sample E (Turkish)	0·048	0·051 0·053	0·049 (Mean of 7 results; range 0·048 to 0·050)

2 minutes add 10 ml. of the ammonium bicarbonate–nickel chloride solution. Dilute to volume with water and mix. To the other flask (the blank) add 5 ml. of 0·1N hydrochloric acid and 10 ml. of the ammonium bicarbonate–nickel chloride solution and mix.

Read the optical density after 90 minutes in a 1 cm. cell at 670 mμ on a suitable spectrophotometer or with an Ilford Filter No. 608 if using a "Spekker" or similar instrument.

Determine the weight of anhydrous morphine present by reference to a calibration curve. Alternatively compare the optical density with that of a complex developed at the same time on a standard solution of morphine of suitable dilution. The results obtained on a number of prepared samples are given in Table I.

Opiate Linctus of Squill B.P.C. Standard samples were prepared by accurately diluting the Camphorated Tinctures of Opium referred to above.

A direct column purification cannot be applied to this preparation because of the large quantity of the sample required. An extraction method which separates the morphine from interfering ingredients without the formation of emulsions is detailed below. Oxymel of Squill and Syrup of Tolu gave no colour when similarly treated. Recoveries of morphine

made in the presence of a "blank" sample were quantitative. The method of assay is as follows.

Transfer 30 ml. of sample to a separator. Add 1 ml. dilute solution of ammonia. Mix. Shake vigorously for 2 to 3 minutes with 30 ml. of the chloroform-*iso*butyl alcohol mixture. Allow to separate and run the solvent phase into another separator containing 10 ml. of water. Shake well and after separation filter the solvent through a plug of cotton-wool into a small beaker. Repeat the extraction in the first separator with two further quantities of chloroform-*iso*butyl alcohol, each of 30 ml., washing

TABLE II

COMPARISON OF THE DETERMINATION OF MORPHINE BY THE B.P.C. METHOD AND THAT OF PRIDE AND STERN IN THREE SAMPLES OF OPIATE LINCTUS OF SQUILL. B.P.C.

	Per cent w/v anhydrous morphine		
	Calculated	B.P.C. method	Proposed method
Sample A (Indian)	0·0156	0·0180	0·0158 (Mean of 13 results; range 0·0153 to 0·0161)
Sample B (Indian)	0·0176	0·0200	0·0177 (Mean of 13 results; range 0·0175 to 0·0180)
Sample C (Turkish)	0·0145	0·0180	0·0146 (Mean of 5 results; range 0·0145 to 0·0147)

successively with the same 10 ml. as before, and filtering into the beaker. Rinse the filter and plug with a little of the solvent. Evaporate the solvent on a steam bath under a current of air and leave the residue on the bath for a further 5 minutes. Cool. Take up the residue in 5 ml. 0·5N hydrochloric acid, accurately measured, warming slightly for a few seconds if necessary. Filter the solution through a Whatman No. 41 filter paper into a 50 ml. graduated flask washing the beaker well with small quantities of water and transferring to the filter. Cool to laboratory temperature if necessary then make up to volume with water.

Continue by the method for Camphorated Tincture of Opium from the words "Take 10 ml. of the solution prepared above. . . ."

Results obtained on a number of samples are given in Table II.

Compound Camphorated Linctus of Opium B.P.C. 1949

Standard samples were again prepared by accurate dilution of the Camphorated Tinctures of Opium.

By the official method some extracted material gives a bright yellow colour on adding ammonia, even in the absence of nitrite. The proposed method separates morphine from other ingredients; a sample made up with all ingredients except Tincture of Opium gave negligible absorption at wavelengths between 400 and 800 mμ.

Transfer 50 ml. of sample to a separator. Add 3 ml. dilute solution of ammonia. Mix well. Shake vigorously for 2 to 3 minutes with 50 ml. of the chloroform-*iso*butyl alcohol mixture. Allow to separate and run the solvent into a 250 ml. separator. Extract the solution in the first

separator with two further portions, each of 50 ml. of chloroform-*iso*butyl alcohol mixture and bulk with the first extract. Reject the aqueous phase. Extract the morphine from the solvent by shaking with two portions each of 10 ml. followed by one portion of 5 ml. of 2 per cent sulphuric acid solution, passing each extract through the same plug of

TABLE III

COMPARISON OF THE DETERMINATION OF MORPHINE BY THE B.P.C. METHOD AND THAT OF PRIDE AND STERN IN TWO SAMPLES OF COMPOUND CAMPHORATED LINCTUS OF OPIUM B.P.C. 1949

	Per cent w/v anhydrous morphine		
	Calculated	B.P.C. 1949 method	Proposed method
Sample A (Indian)	0·0143	0·0190	0·0145 (Mean of 4 results; range 0·0144 to 0·0146)
Sample B (Turkish)	0·0125	0·0180	(Mean of 4 results; range 0·0126 to 0·0128)

cotton-wool into another separator. Wash the filter with a few drops of water and make the bulked acid extracts alkaline to litmus paper with dilute solution of ammonia. Continue by the method for Opiate Linctus of Squill beginning at the words "Shake vigorously for 2 to 3 minutes with 30 ml. of the chloroform-*iso*butyl alcohol mixture. . . ." Results obtained on several samples are given in Table III.

TABLE IV

COMPARISON OF THE DETERMINATION OF MORPHINE BY THE B.P.C. METHOD AND THAT OF PRIDE AND STERN IN TWO SAMPLES OF AMMONIATED TINCTURE OF OPIUM B.P.C. 1949

	Per cent w/v anhydrous morphine		
	Calculated	B.P.C. 1949 method	Proposed method
Sample A (Indian)	0·100	0·115	0·099 (Mean of 6 results; range 0·097 to 0·103)
Sample B (Indian)	0·096	0·112	0·097 (Mean of 5 results; range 0·096 to 0·098)

Ammoniated Tincture of Opium B.P.C. 1949

Samples were prepared from standard Tinctures of Opium. The procedure adopted for the assay of Camphorated Tincture of Opium may be applied since benzoic acid and oil of anise do not interfere. Because of its high pH the sample is made just acid before evaporation, to avoid possible oxidation of the morphine. The recommended method is as follows.

Take 5 ml. of sample in a small evaporating dish and add 3N sulphuric acid dropwise with stirring until the mixture is just acid to litmus paper. Evaporate the solution to dryness on a steam bath and continue by the method for Camphorated Tincture of Opium beginning at the words "Triturate the residue with 1 ml. dilute solution of ammonia". Results obtained are given in Table IV.

Opiate Pastilles of Squill B.P.C.

Since it was not possible to prepare pastilles containing an accurately known amount of morphine tests were made on production batches. Different methods of extracting the morphine and of preparing solutions for colour formation were used and where the solution had a pH below the critical value of 1·6 colours were developed and extinction values

TABLE V

COMPARISON OF THE DETERMINATION OF MORPHINE BY THE B.P.C. METHOD AND THAT OF PRIDE AND STERN IN TWO SAMPLES OF PASTILLES OF OPIATE LINCTUS OF SQUILL B.P.C.

	Anhydrous morphine per pastille in g.	
	B.P.C. method	Proposed method
Batch 2374	0·00036	0·00029 (Mean of 5 results; range 0·00029 to 0·00030)
Batch 2493	0·00037	0·00028 (Mean of 3 results; range 0·00027 to 0·00028)

measured. The method yielding the optimum recovery of morphine and a minimum of interference from large excesses of other ingredients are as follows.

Take a sample of 20 pastilles and determine the average weight. Cut up some and accurately weigh into a separator an amount equivalent to about 5. Add 30 ml. of water, stopper the separator and shake vigorously to dissolve. Make the solution alkaline with dilute solution of ammonia

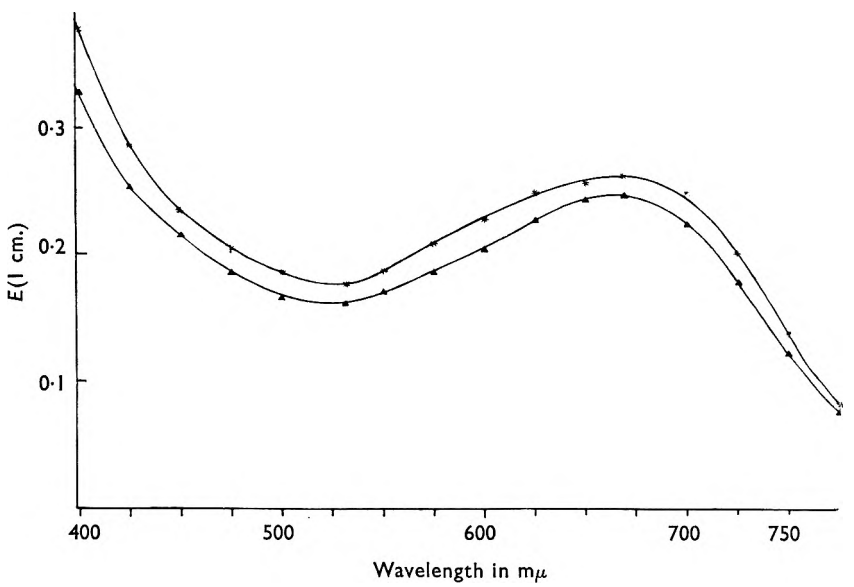

FIG. 2. Absorption curves of colours developed from morphine standard and that obtained from Tincture of Chloroform and Morphine B.P.C.
—*—*— Morphine standard.
—▲—▲— Tincture of Chloroform and Morphine B.P.C.

and extract with three successive portions, each of 30 ml. of chloroform-*iso*butyl alcohol mixture shaking well at each extraction for 2 to 3 minutes. Collect the extracts in a second separator and reject the aqueous phase. Extract the solvent mixture with two successive portions, each of 10 ml. followed by one of 5 ml. of 2 per cent sulphuric acid solution, bulking all three acid extracts in the same separator. Make ammoniacal and continue by the method for Opiate Linctus of Squill beginning at the words "Shake vigorously for 2 to 3 minutes with 30 ml. of the chloroform-*iso*butyl alcohol mixture". Read the optical density of the green complex in a 4 cm. cell.

Results obtained on production batches sampled at random are given in Table V.

Tincture of Chloroform and Morphine B.P.C.

That the nitroso-phenol reaction when applied to extractives of chlorodyne is subject to interference from other substances present in the residue has been known for some time. Garratt[21] in 1946 drew attention

TABLE VI

COMPARISON OF THE DETERMINATION OF MORPHINE BY THE B.P.C. METHOD AND THAT OF PRIDE AND STERN IN THREE SAMPLES OF TINCTURE OF CHLOROFORM AND MORPHINE B.P.C.

	Per cent w/v anhydrous morphine			
			Proposed method	
	Calculated	B.P.C. method	Sample alone	With 5-fold excess of Liquorice
Sample 1 (Liquid Extract of Liquorice P9216)	0·174	0·190	0·175 (Mean of 11 results; range 0·174 to 0·179)	0·174
Sample 2 (Liquid Extract of Liquorice 1872M)	0·177	0·210	0·176 Mean of 5 results; range 0·173 to 0·176)	0·176
Sample 3 (Liquid Extract of Liquorice P9240)	0·174	0·192	0·172 (Mean of 4 results; range 0·168 to 0·175)	0·174

to apparent "morphine" contents of treacle. In 1951 McLachlan[22] reported the interference from liquorice as being due to the colour which develops with ammonia and glycyrrhizin and some of its hydrolysis products. In 1956 the "apparent morphine contents" of both treacle and liquid extract of liquorice were determined in the laboratory of the Pharmaceutical Society[17], and high recoveries of morphine were obtained by application of the Pride and Stern reaction.

By the extraction procedure detailed below morphine can be effectively separated from the substances present in liquorice which give a colour with ammonia: no yellow colour was developed on addition of the strongly ammoniacal complexing reagent to the blank solution.

Determinations on blank mixtures containing all ingredients except morphine hydrochloride gave negligible extinction values. No colour was obtained on treating Liquid Extract of Liquorice B.P. by the same

procedure. Figure 2 shows absorption curves for the colours obtained from a morphine standard and from chlorodyne. Quantitative recoveries of morphine were obtained using a number of different batches of Liquid Extract of Liquorice, each present in a five-fold excess. For recorded figures obtained on samples prepared from morphine hydrochloride standardised by the fluorodinitrobenzene method, see Table VI. The recommended method of assay is as follows.

Determine the weight/ml. of the sample.

Accurately weigh about 4 g. of sample into a small dish and evaporate the volatile solvents on a steam bath. Cool. Triturate the residue to a smooth cream with 1 ml. dilute solution of ammonia and continue by the method for Camphorated Tincture of Opium beginning at the words "add aluminium oxide gradually . . ." and ending at ". . . adjusting the rate of elution to about 1·5 ml. per minute". Extract the morphine from the eluate with two successive portions each of 10 ml. followed by one of 5 ml. of 2 per cent sulphuric acid solution. Bulk the acid extracts in another separator. Make alkaline to litmus paper with dilute solution of ammonia and proceed by the method for Opiate Linctus of Squill beginning at the words "shake vigorously for 2 to 3 minutes with 30 ml. of the chloroform-*iso*butyl alcohol mixture".

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DISCUSSION

The paper was presented by MR. C. A. JOHNSON.

The CHAIRMAN. The nitrosomorphine method had not always been satisfactory, often because of impurities in the final reaction mixture. Better methods of separation of the morphine might improve the results. Had the authors applied the nitrosomorphine method to their final reaction mixtures?

MR. S. G. E. STEVENS (London). How had the Authors arrived at the 'calculated' figures in the Tables?

DR. A. H. BECKETT (London). Was it possible to determine morphine in biological fluids by this method and would it then distinguish between morphine and normorphine?

DR. G. F. SOMERS (Liverpool). How many assays were represented by the figures quoted for the B.P. and B.P.C. assays?

DR. R. E. STUCKEY (London). Recoveries from pastilles were often poor but the authors' method had given a slightly lower figure than the B.P.C. method and no theoretical figure was stated.

MR. JOHNSON replied. 'Calculated' referred to samples prepared on a laboratory scale. The nitrosomorphine reaction had been applied to solutions prepared as described in the Paper and showed a great improvement, but there were other objections to the method. Normorphine had been shown by Pride and Stern to give less colour than morphine. If the two occurred together there would be some interference. The results quoted in the second columns of the Tables were supported by experience over many years. The figures for the pastilles were included to indicate that the proposed method gave lower results than the official method.

SOME OBSERVATIONS CONCERNING THE CHEMICAL REACTIONS OCCURRING BETWEEN FORMALDEHYDE AND PEPTONE

By Kenneth Bullock and V. Subba Rao*

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The uptake of formaldehyde by peptone has been studied. Relatively stable powders (formol-peptones) can be prepared by exposing peptone powder to the vapour phase over formalin for four days and drying the product *in vacuo*. Different batches of such powders, obtained from the same sample of peptone are of similar composition but formol-peptones obtained from different makes of peptone may differ considerably in composition. The state of binding between the formaldehyde and the peptone in powder and in solution has been investigated by means of the chromotropic acid and Vorländer's reactions. The firmness with which formaldehyde is bound to peptone in solution depends not only on the quantities of formaldehyde and peptone present but also on the previous relationship of the two substances. The reactions between formaldehyde and peptone have been found to be complex. Equilibrium is not readily attained either in solution or when peptone powder is exposed to formalin vapour.

It has been known for a long time that the disinfectant activity of such substances as formaldehyde (HCHO) is reduced by the presence of organic matter such as peptone. Bullock and Rawlins found that during the spray-drying process while 0·02 per cent formaldehyde ensured a sterile product in the absence of peptone, 0·4 per cent was necessary in the presence of peptone[1]. In later work, when studying the disinfection of powders by formaldehyde vapour, it was found that even small amounts of peptone reduced considerably the effectiveness of the HCHO[2]. Since formaldehyde is used as a disinfectant both in solution and as vapour it was decided to investigate the reactions occurring between peptone powder and formaldehyde vapour as well as the reactions occurring in solution and if possible, to relate the types of combination between these two substances with the disinfectant power of the products. These observations are the subject of a separate report[3].

The subject has interest because of the use of HCHO in the preparation of toxoids as well as its use as a disinfectant in solution and as vapour in the sterilisation of surfaces and powders.

Experimental

Peptone. Three commercial brands of peptone A, B and C were used. A fourth powder D was obtained by dissolving a quantity of A in water and spray-drying the solution. A comparison of powders A and D was used to determine whether the physical characteristics of a spray-dried powder exerted any effects. Since no such effects were observed no further reference to powder D will be made.

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Solution of Formaldehyde B.P. (Formalin) found to contain 38·2 per cent HCHO by the B.P. assay process was used in the preparation of formol-peptones and after dilution and neutralisation to phenolphthalein, in the formol titrations.

Solution of Formaldehyde A.R. (Formalin A.R.) found to contain 38·4 per cent HCHO by the B.P. assay process was used in all other cases.

Chromotropic acid and dimedone (5 : 5-*dimethyl*cyclo*hexane*-1 : 3-*dione*) were both of commercial quality (B.D.H.).

Formol-peptone was prepared by placing a quantity of formalin in the bottom of a desiccator and above it a thin layer of peptone on a clock glass. After four days in the closed desiccator, the peptone, now a sticky paste, was transferred to a second desiccator and dried over P_2O_5 under reduced pressure. After one week's drying the resultant "formol-peptone" was powdered. Such formol-peptones were prepared from peptones A, B and C.

Carbonate free alkali. Solutions of sodium hydroxide for use in titrations were freed from carbonate by the addition of barium chloride and filtration before standardisation with oxalic acid (phenolphthalein indicator).

Chromotropic acid reagent[2]. 0·2 g. chromotropic acid + 20 ml. water was filtered and 80 ml. sulphuric acid solution (2 volumes sulphuric acid A.R. + 1 volume water) added to the filtrate; it was stored in a stoppered bottle protected from light and was prepared freshly each week.

Vorländer's Reagent[4] (*dimedone solution*). 0·2 g. dimedone in 100 ml. of McIlavaine buffer (22·7 ml., 0·2M sodium phosphate + 2·3 ml., 0·1M citric acid) was prepared freshly each week.

Methods

Neutralising titration values (*N.T.Vs.*) *and formol titration values* (*F.T.Vs.*). 10 ml. of peptone solution, containing approximately 100 mg. peptone, was titrated with 0·1N NaOH to a pink colour with phenolphthalein to obtain the neutralising titre. 10 ml. of neutral HCHO solution, equal parts of formalin and water neutralised to phenolphthalein, was then added and the mixture again titrated with 0·1N NaOH to a pink colour to obtain the formol titration titre. The titres were used to calculate the number of ml. of 0·1N NaOH required by 1 g. of the original peptone for neutralisation before (N.T.V.) and after addition of the HCHO (F.T.V.). These values were used by Bullock and Sen in their work on papain[5] and the tryptic activity of pancreatin[6].

Determination of formaldehyde by the chromotropic acid reagent[2,7]. 1 ml. of test solution, containing up to 12 μg. HCHO, was mixed with 9 ml. of chromotropic acid reagent, heated at boiling water bath temperature for 30 minutes and the resultant purple colour evaluated in a Spekker photoelectric absorptiometer using a combination of blue (OB_2) and orange (OY_2) filters and as blank a solution obtained by repeating the above process using 1 ml. of water in place of 1 ml. HCHO solution. There was a practically linear relation between the quantity of HCHO

and the colour developed under the conditions. A calibration curve was used to convert Spekker readings into μg. of HCHO in subsequent assays.

Determination of chemically free and chemically bound HCHO by Vorländer's Reagent[4]. 2 ml. of test solution, containing up to 24 μg. total HCHO, was mixed with 2 ml. of Vörlander's reagent and held at 37° for 30 minutes[7]. HCHO was determined in 1 ml. of this mixture by chromotropic acid reagent as described above. The HCHO estimated in this way, that is that strongly enough bound to peptone to resist combination with Vorländer's but nevertheless capable of reacting with

TABLE I

PERCENTAGE OF HCHO, ADDED TO WATER AS FORMALIN, WHICH COMBINES WITH VÖRLANDER'S REAGENT

Experiment number	HCHO added mg.	HCHO reacting with CA in presence of VR, mg.	HCHO combining with VR, mg.	HCHO combining with VR per cent
1	52·0	1·4	50·6	97·3
2	49·1	0·9	48·2	98·2
3	99·2	3·6	95·6	96·4
4	227·2	6·4	220·8	97·2
5	164·0	3·1	160·9	98·1

CA = Chromotropic acid reagent.
VR = Vörlander's reagent.

chromotropic acid reagent, will be referred to below as bound HCHO (V.R.) Free HCHO (V.R.) was determined as the difference between the figures for HCHO by chromotropic acid reagent in the presence and in the absence of Vorländer's reagent. Macfadyen[7] found that only 98·5 per cent of free HCHO is fixed by dimedone. Table I shows that under the conditions used in this work when a dilution of formalin A.R. is examined by the Vorländer reaction about 97·4 per cent is returned as "free" and 2·6 per cent as "bound". Making the assumption that such a dilution contains only free HCHO all experimental figures were adjusted to allow for this finding before being recorded as free HCHO (V.R.).

RESULTS

The reactions between peptone and formaldehyde have been found to be complex. Not only is there a difference in the ways in which formaldehyde reacts with the peptone when, on the one hand both are in solution, or, on the other hand, when the peptone is as powder and HCHO as it occurs in the vapour phase over formalin, but even under constant environmental conditions the changes are progressive and dependent on the degree of access of moisture vapour or water. Three types of changes have been followed. (1) Changes when (*a*) peptone powder is exposed to the vapour phase over formalin, and when (*b*) the products are dissolved in water. (2) Changes when the dried products of (1), i.e., formol-peptones are dissolved in water. (3) Changes in peptone solutions to which are added quantities of HCHO similar to those present in the formol-peptones.

(1) *Changes in neutralising and formol titration values when* (a) *peptone powder is exposed to the vapour phase over formalin, and* (b) *further changes in these values when the products are dissolved in water.* (*a*) The N.T.Vs. of peptones A, B and C were found to be 10·5, 14·2 and 3·3 respectively,

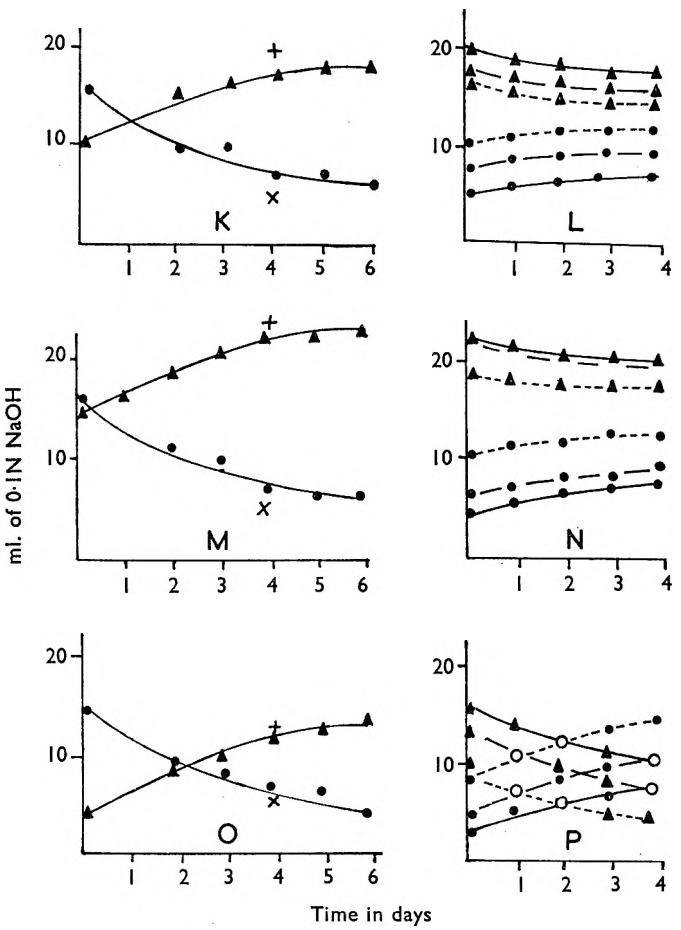


FIG. 1. Changes with time in the neutralising titration values (N.T.V. ▲ ▲) and formol titration values (F.T.V. ● ●) of (*a*) peptones A, B and C when exposed to the vapour phase over formalin (graphs K, M and O respectively) (*b*) solutions of the resultant pastes after exposure for 2 days (– – – – L, N and P) and for 4 days (— — L, N and P) (*c*) solutions of the corresponding formol-peptones (—— L, N and P).

In K, M and O, + represents the N.T.V. and × the F.T.V. of the corresponding formol-peptone.

the corresponding F.T.Vs. being 16·0, 15·4 and 14·9. Assuming that one equivalent of alkali corresponds to one equivalent of nitrogen these figures correspond to 2·24 per cent, 2·16 per cent and 2·09 per cent of amino nitrogen in the corresponding peptones.

Two gram quantities of peptone were, as described above, exposed in a desiccator to the vapour phase over formalin. The N.T.Vs. and F.T.Vs. of the resultant sticky masses were determined after two, three, four, five and six days' exposure in the desiccator. For comparative purposes and to avoid complications due to the moisture content of the peptones, the values were expressed per gram of original peptone. The results for peptones A, B and C are graphed in Figure 1, K, M and O.

(*b*) When the resultant sticky masses were dissolved in water the N.T.Vs. and F.T.Vs. of the solutions changed slowly over the first three or four days and then became constant for some time. These changes are shown in Figure 1, L, N and P.

(2) The N.T.Vs. and F.T.Vs. of formol-peptones A, B and C are shown marked × and + respectively in Figure 1, K, M and O. It was found that different batches of formol-peptone prepared from the same sample of peptone were of similar composition. It can be seen from the × and + marks in Figure 1 that the formol-peptone C differed considerably in composition from formol-peptones A and B (see also Fig. 2). The changes which occurred in the first four days when these formol-peptones were dissolved in water are graphed in Figure 1, L, N and P. In an experiment which lasted ten days the values became practically constant after four days.

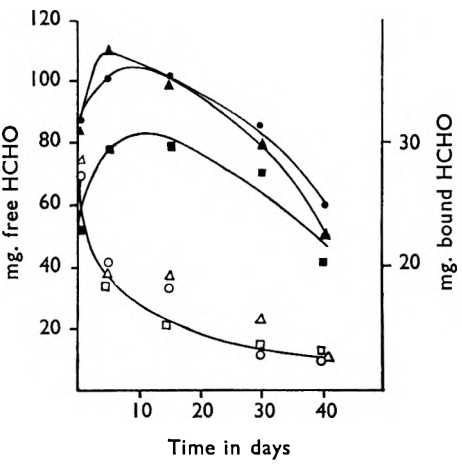


FIG. 2. Changes in bound HCHO and free HCHO on storage of solutions of formol-peptones.

Free HCHO			Bound HCHO		
Peptone	A	▲	Peptone	A	△
,,	B	●	,,	B	○
,,	C	■	,,	C	□

Chemically bound and chemically free formaldehyde in solutions of formol-peptones. There is a sense in which all the HCHO in formol-peptone could be considered to be bound either physically or chemically since the final step in the preparation was a six day storage *in vacuo*. There is also a sense in which the state of the HCHO in formol-peptones cannot be determined by the chemical methods described above, since, in such methods, aqueous solutions are used and, as shown below, as soon as formol-peptones are exposed to either water vapour or water, changes in the state of combination of the HCHO set in. However, for the present purposes, it will be considered that the state of chemical binding of the HCHO in a formol-peptone can be judged by examining a freshly prepared solution.

Calculation of chemically bound formaldehyde from F.T.Vs. When formaldehyde reacts with peptone it must react with some chemically active centres, generally considered to be free-NH_2 groups. If, therefore, a small quantity of HCHO is added to some peptone the F.T.V. of the latter will decrease in proportion to the number of receptors blocked and therefore it is reasonable to consider the drop in F.T.V. to be a measure of the chemically bound formaldehyde. If the F.T.V. of peptone in a solution containing HCHO increases, it indicates that some of the HCHO previously bound to the peptone has become free. If the F.T.V. decreases then conversely some of the HCHO has become bound to the peptone. By making these assumptions it is possible to calculate the bound HCHO in formol-peptones. Thus for peptone A:—

F.T.V. original peptone = 16·0
F.T.V. formol-peptone A = 4·3

Difference = 11·7 ml. 0·1N NaOH

but 1 ml. 0·1N NaOH in the formol titration is ≡ 3 mg. HCHO
∴ 11·7 ml. 0·1N NaOH ≡ 35·1 mg. HCHO

Thus a quantity of formol-peptone A equivalent to 1 g. of original peptone A contained 35·1 mg. bound HCHO. The corresponding quantities for formol-peptones B and C were 33·2 mg. and 25·2 mg. respectively.

In the following discussion bound formaldehyde determined in this way is referred to as bound HCHO (F.T.V.). Changes in the F.T.Vs. of solutions of formol-peptones can thus be expressed as changes in bound HCHO (F.T.V.). It was found that the values for bound HCHO (F.T.V.) were about 10 per cent and 20 per cent higher than bound HCHO (V.R.) for formol-peptones A and B respectively. The two values were about the same for formol-peptone C.

The changes in free HCHO (V.R.) and bound HCHO (V.R.) which occur when formol-peptone solutions are stored are shown in Figure 2.

The fate of HCHO added as Formalin A.R. to 1 per cent peptone solutions. It was decided that it would be useful to compare the difference in the mode of combination between HCHO and peptone when the former is (*a*) taken up from the vapour phase over formalin as in the preparation of formol-peptone and (*b*) added as formalin A.R. to 1 per cent peptone solution. To do this the above results with formol-peptone solutions were compared with similar results obtained by storing 1 per cent peptone solutions to which had been added quantities of HCHO corresponding to the quantities of total HCHO estimated to be present in the corresponding 1 per cent formol-peptone solutions. Thus, in 1 per cent solutions of peptones A and B were incorporated quantities of formalin A.R. corresponding to 0·1 per cent HCHO while to the 1 per cent solution of peptone C, 0·06 per cent HCHO was added.

The results of submitting such solutions, immediately on preparation and after storage, to the usual analytical procedure is shown in Figures 3 and 4. These should be compared with Figures 1 and 2.

Manner of the uptake of HCHO by peptone from the vapour phase over formalin. The increase in weight of the peptone on conversion to formol-peptone falls between that required for the simple addition of HCHO (M.Wt. = 30) and that required for this followed by elimination of 1 molecule of water (HCHO − H_2O = 30 − 18 = 12). Using peptone

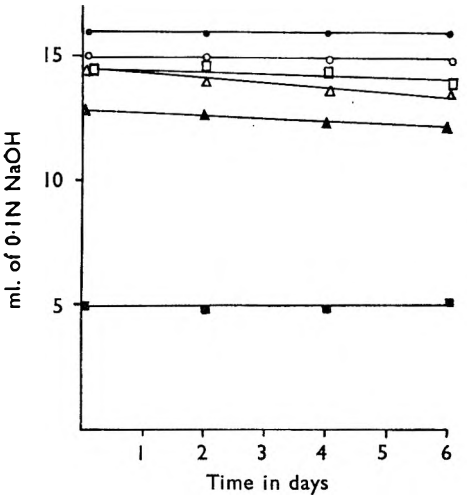


FIG. 3. Changes with time in N.T.Vs. and F.T.Vs. when to 1 per cent solutions of peptones A, B and C are added 0·1 per cent, 0·1 per cent and 0·06 per cent respectively of HCHO.

Peptone	N.T.V.	F.T.V.
A	▲	△
B	●	○
C	■	□

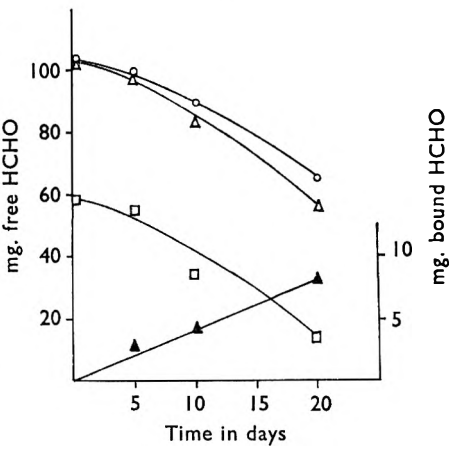


FIG. 4. Changes in free HCHO and bound HCHO in 1 per cent aqueous solutions of peptones A, B and C.

	HCHO Added	Free	Bound
A	0·1 per cent	△	▲
B	0·1 ,,	○	▲
C	0·06 ,,	□	▲

A as an example, the following calculation illustrates the way in which the results shown in Table II were obtained.

1·9564 g. dry peptone gave 2·1022 g. formol-peptone showing an increase of 0·1458 g. due to uptake of HCHO. But by the chromotropic acid reagent method the formol-peptone contained the equivalent of 0·1879 g. HCHO. If therefore the HCHO had been taken up without loss of water the increase in weight would have been 0·1879 g. On the other hand if for every molecule of HCHO added 1 molecule of water had been lost the increase in weight would have been $0{\cdot}1879 \times \frac{12}{30} = 0{\cdot}07516$ g.

Increase in weight with no loss of water = 0·1879. Increase in weight with 100 per cent loss of water = 0·0752. Difference = 0·1127. Increase in weight with no loss of water = 0·1879. Increase in weight found = 0·1458. Difference = 0·0421. Therefore the percentage of the reactions occurring with loss of water = $100 \times \frac{0{\cdot}0421}{0{\cdot}1127} = 37$. Therefore the percentage of the reactions occurring without loss of water = 63.

Storage of formol-peptone. Two samples of formol-peptone powder A were stored for 45 days. The first in a desiccator over P_2O_5, lost only 3·7 per cent of its total HCHO content (chromotropic acid method). The powder remained dry and free flowing. The second sample which was stored in the laboratory in a bottle closed with a cotton wool plug lost 28 per cent of its total HCHO. The powder caked due to uptake of moisture. In an attempt to ascertain whether the decrease in HCHO content of the formol-peptone in the second sample was due to release and volatilisation of HCHO resulting from moisture uptake, a thin layer

TABLE II

DATA FOR CALCULATING THE PERCENTAGE OF THE REACTIONS BETWEEN HCHO AND PEPTONE INVOLVING ELIMINATION OF WATER

Peptone	Weight taken	Weight after drying	Weight of resultant formol-peptone	Increase in weight due to uptake of HCHO	HCHO content by CA	Percentage of reactions not involving elimination of H_2O
A	2·0572	1·9564	2·1022	0·1458	0·1879	63
B	2·0062	2·0056	2·2056	0·2000	0·2456	69
C	2·0900	2·0758	2·2700	0·1942	0·2268	76

TABLE III

LOSS OF HCHO FROM FORMOL-PEPTONE POWDER WHEN STORED OVER : (*a*) 0·1N $K_2Cr_2O_7$ + H_2SO_4 AND (*b*) SOLID $K_2Cr_2O_7$ + H_2SO_4. FIGURES GIVE THE LOSS OF HCHO AS PER CENT OF THE TOTAL PRESENT (CA METHOD)

(*a*)			(*b*)		
Time of storage, days	Loss by CA	Loss by $K_2Cr_2O_7$ reduction	Time of storage, days	Loss by CA	Loss by $K_2Cr_2O_7$ reduction
4	57	62	7	4·0	10·0
6	62	64	30	4·5	11·5

of formol-peptone was stored over (*a*) acidified 0·1N $K_2\ Cr_2\ O_7$ and (*b*) solid $K_2\ Cr_2\ O_7$ moistened with strong sulphuric acid. The results are shown in the Table III.

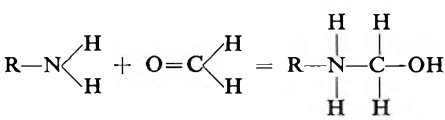
DISCUSSION

It is apparent from Figure 1, K, M and O that in addition to water, peptone powder absorbed HCHO when exposed in a thin layer to the vapour of formalin; the N.T.V. rose and the F.T.V. fell. The sum of these two values remained approximately constant. Presumably the HCHO in the vapour phase in the presence of the moisture reacted with the peptone in a manner comparable to that in which addition takes place during a formol titration. The drop in F.T.V. was at first rapid then it slowed but was not complete after six days. The products became increasingly less soluble in water during the six days. When the resultant sticky masses were dissolved in water there was an initial slight reversal of the changes which had been progressing. As shown in Figure 1, L, N and P, the N.T.Vs. fell and the F.T.Vs. rose, these changes being progressive for four days when the values remained approximately constant for the next six days. Taking all these facts into consideration it was decided

to prepare the formol-peptones by drying the peptone pastes after four days exposure to the vapour phase over formalin. One would have expected that when the pastes were removed from the formalin vapour and placed over P_2O_5 under reduced pressure the uptake of HCHO would have ceased or even been reversed. However, from Figure 1, K, M and O it can be seen that the N.T.V. (marked +) has increased and the F.T.V. (marked ×) has decreased beyond the values to be expected from exposure for four days. The pastes presumably contained free HCHO which continued to react with the peptone. It should be mentioned that when the pastes were exposed to reduced pressure they frothed to a considerable extent due to the vapourisation of the water and free HCHO. This considerably facilitated the drying process and resulted in a porous, easily powdered, solid residue being obtained. In spite of these facts and in spite of the fact that the dried and powdered formol-peptones were left over P_2O_5 under reduced pressure for six days, when the formol peptone powders were dissolved in water to give a 1 per cent solution the N.T.Vs. fell and the F.T.Vs. rose in a manner which paralleled the changes occurring when the corresponding pastes were similarly dissolved in water. Presumably the equilibrium between HCHO and peptone is different in pastes and in the 1 per cent solutions of the peptone. When 1 per cent formol-peptone solutions were stored, as shown in Figure 2, the bound HCHO (V.R.) fell, at first rapidly, then with increasing slowness. There was, during the first six days, a rise in free HCHO (V.R.) which was followed by a fall which became increasingly rapid. The fall was probably due to loss of HCHO by volatilisation from the solution. It was observed that the solutions, after one month became turbid due to multiplication of adventitious micro-organisms. Initially the free HCHO rose to a greater extent than the bound HCHO fell. This was due to the fact that the total HCHO as judged by the chromotropic acid method rose. During the first six days the rise in total HCHO calculated per gram of original peptone was 15·9 mg., 7·7 mg. and 18·0 mg. respectively for formol-peptones A, B and C. Presumably in formol-peptone some HCHO is bound so firmly that it no longer reacts with chromotropic acid; some of this HCHO becomes reactive again in the 1 per cent solution.

A comparison of Figures 3 and 4 with Figures 1 and 2 shows that in solutions of identical composition the strength of binding between HCHO and peptone may vary. In 1 per cent formol-peptone solution the HCHO is largely bound, in a 1 per cent solution of the same peptone a corresponding quantity of HCHO added as formalin A.R. is mainly free and remains mainly in the free state until it can no longer be detected—the free HCHO falling from the beginning and continuing to fall with increasing rapidity.

Nitschmann and Hadorn[8] suggested that when formaldehyde vapour was taken up by casein the HCHO was first bound as "methylol radical".



The methylol radicals then condensed with adjacent reactive groups with the elimination of water forming methylene bridges.

An attempt was made to ascertain whether similar reactions occurred between peptone and HCHO. It appeared (Table II) that while in some cases water is eliminated, in the majority of cases the HCHO and peptone react without loss of water. It must, however, be emphasised that the methods used in the determinations on which the calculations are based are liable to considerable error.

Even when formol-peptone powder is stored over concentrated H_2SO_4 and solid $K_2Cr_2O_7$ reducing vapours (HCHO) are evolved to a slight extent (Table III). When stored over 0·1N $K_2Cr_2O_7$ acidified with sulphuric acid, the powders take up water and evolve HCHO to a considerable extent. It is obvious that in all experiments where the disinfectant effects of HCHO are being studied the presence or absence of water must be taken into account.

Perhaps the most interesting conclusion which can be drawn from the present work is that the interactions between HCHO and peptone are complex and do not readily reach equilibrium.

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THE EFFECTS OF ADDED PEPTONE ON THE BACTERICIDAL ACTION OF SOLUTIONS OF FORMALDEHYDE

By Kenneth Bullock and V. Subba Rao*

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When formaldehyde (HCHO) is used as a disinfectant in the presence of peptone the peptone and the bacteria compete for the HCHO[1]. An equilibrium distribution of the HCHO is reached only slowly. The state of combination of HCHO in the presence of 1 per cent peptone

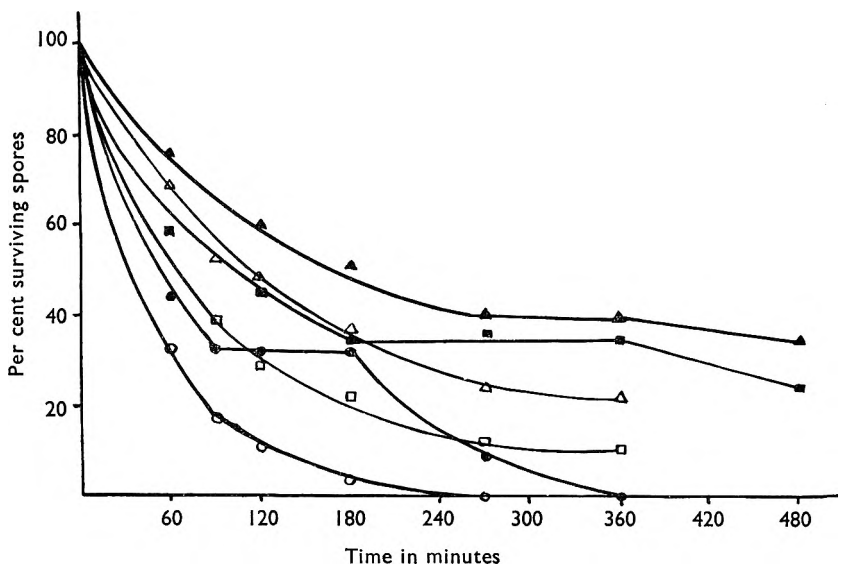

Fig. 1. The influence of the presence of peptone on the survival of *B. subtilis* spores in dilute solutions of HCHO.

A ○ 0·1M HCHO at 37°.
B □ 0·05M HCHO at 37°.
C △ 0·1M HCHO at 25°.
D ● 0·1M HCHO + peptone at 37°.
E ■ 0·05M HCHO + peptone at 37°.
F ▲ 0·1M HCHO + peptone at 25°.

varies with its previous relationship to the peptone. If, in all states of combination with peptone, HCHO were fully available to act as a disinfectant peptone would neither retard nor reduce such action. Estimates of the proportions of HCHO in three different degrees of binding with peptone having been made we now enquired in which of these the HCHO remained bactericidal.

The methods of preparing *B. subtilis* spore suspensions and making roll-tube viable counts have been described[2]. All the solutions containing HCHO were brought to the required temperature and 1 ml. of stock

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spore suspension added to 9 ml. of test solution. After thorough mixing 1 ml. was removed and serial dilutions prepared for roll tube viable counts, from which results the initial count (usually about 20,000/ml.)

TABLE I

EFFECTS OF THE INCREASED BINDING OF HCHO IN FORMOL-PEPTONE ON ITS DISINFECTANT ACTIVITY

Time in minutes	Percentage surviving spores		
	One per cent peptone with added formalin		Formol-peptone 0·1 M HCHO
	0·1 M HCHO	0·05 M HCHO	
65	34	55	40
180	34	34	29
245	15	34	26
480	0	24	0

could be calculated. The desired quantity of HCHO was now added and the whole mixed. After suitable time intervals 1 ml. quantities of the mixture were removed for viable counts of the surviving organisms. The results were expressed as the percentage of the spores remaining viable.

Curves A and B of Figure 1 demonstrate the bactericidal effects of 0·1M and 0·05M HCHO respectively in water at 37°. Curve C was obtained

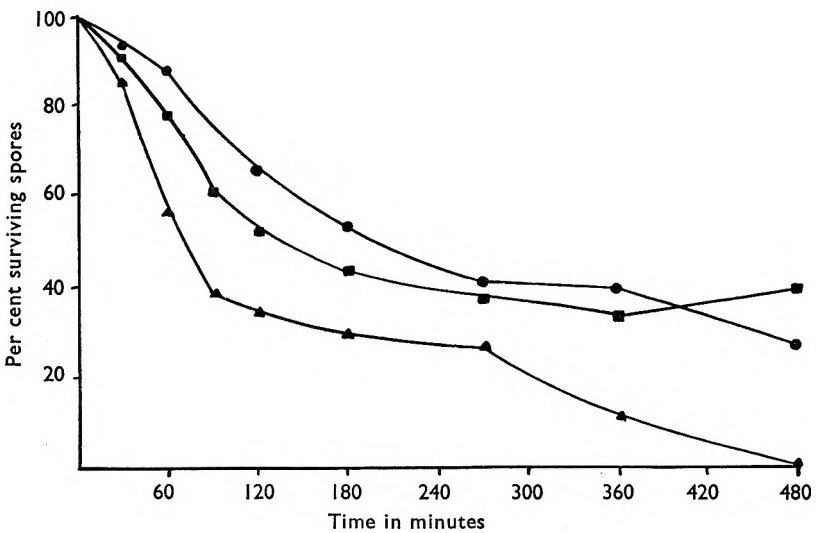

FIG. 2. The survival of *B. subtilis* spores in formol-peptone solutions.
H ▲ ▲ 0·1M HCHO at 37°.
J ● ● 0·1M HCHO at 25°.
K ■ ■ 0·05M HCHO at 37°.

with 0·1M HCHO at 25°. Curves D, E and F represent corresponding experiments in the presence of 1 per cent peptone. Increasing the peptone content from 1 to 5 per cent had no effect. Attempts were made to explain the flat portion of curve D. If the peptone and HCHO were

kept at 37°, for 3 hours before addition of the spores the shape of the curve was unaltered. If the peptone solution and spores were incubated at 37° for 3 hours before the addition of HCHO all the spores were killed in 60 minutes by 0·1M HCHO and in 90 minutes by 0·05M HCHO. Incubation in nutrient solution is known to induce, in a very short time, incipient germination of spores, possibly such germination, even in the

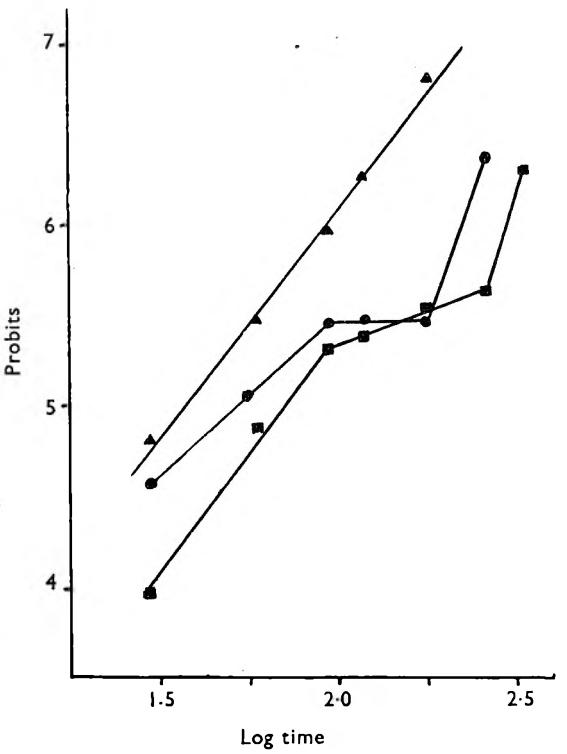

FIG. 3. Probit log-time regression curves of the survival of *B. subtilis* spores.
L ▲ ▲ 0·1M HCHO at 37°.
M ● ● 0·1M HCHO + peptone at 37°.
N ■ ■ 0·1M HCHO from formol-peptone at 37°.

presence of low concentrations of HCHO might explain the broken curves of Figure 1.

In further experiments such quantities of formol-peptones were dissolved in water that the resultant solutions contained 0·1M or 0·05M total HCHO estimated by chromotropic acid. Peptone was added, if necessary, to bring its concentration to 1 per cent. Time survivor curves for spores in such solutions are shown in Figure 2. Figure 3 shows the probit-log time regression curves drawn from the data used to construct curves A and D (Fig. 1) and H (Fig. 2). In Table I the action of 0·1M HCHO derived from formol-peptone is compared with that of 0·1M and 0·05M HCHO added as a dilution of formalin to 1 per cent peptone solution.

The increased binding of the HCHO to the peptone, previously demonstrated in the formol-peptone solutions, has surprisingly little effect on its disinfectant action. Most of the HCHO bound to the peptone sufficiently firmly to resist removal under reduced pressure in the dry state was bactericidal when the formol-peptone was dissolved in water. Even the HCHO bound to the peptone so firmly that it was not transferred to dimedone in solution appeared to possess some, even if a reduced, bactericidal activity.

Acknowledgement. One of the authors (V. S. R.) is a recipient of a Modified overseas scholarship of the Government of India.

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DISCUSSION

The Paper and Short Communication were presented together by PROFESSOR K. BULLOCK.

The CHAIRMAN. Could sodium bisulphite combine with bound as well as free formaldehyde?

DR. F. HARTLEY (London). According to Table I in the Short Communication the presence of peptone did not modify the disinfectant properties of formaldehyde to the same extent as previously reported. Was this because moisture was present?

MR. G. SYKES (Nottingham). It was possible to draw a straight line through the black triangles, dots and squares of Figure 1 of the Communication. Only 7 points were recorded from which a curve, a straight line and a curve had been created. Instead of a flat portion, the curve should dip away very quickly, more regularity in the change would be expected and for the curve to become suddenly flat was against the rules of the distribution of resistance in a bacterial population. The N.T.V. of a peptone was not connected with its composition, it was dependent on the acidity or alkalinity at which it was made. Since the summations of F.T.V. and N.T.V. for A, B and C were the same, there was no apparent difference between them according to these estimations.

MR. H. D. C. RAPSON (Betchworth). Even the reaction between formaldehyde and simple amino acids is complex and may involve NH groups as well as NH_2. Formaldehyde may possibly react with hydrogen bonding in a protein and in addition there was the possibility of aldol condensation. Had the Authors considered the technique of vapour phase titration?

DR. A. H. BECKETT (London). Under controlled pH the NH_2 group of proteins was converted to CH_2OH. In work on bacterial surfaces he had found that formaldehyde could block NH_2 groups.

DISCUSSION

DR. J. B. STENLAKE (Glasgow). Acid amide groups react with formaldehyde and primary and secondary amines in Mannich type reactions. Some of the links formed in the Authors' experiments were possibly of this type and the products would be labile. Infra-red techniques might indicate the type of link. The release of water could be accounted for by Mannich type links. Dehydration resulting in azomethane links was also probable. Investigation by end-absorption or ultra-violet techniques might be helpful.

PROFESSOR BULLOCK replied. Sodium bisulphite had not been used. The formal peptones had a higher ratio of formaldehyde than those in previous work. The slightest trace of moisture greatly increases the disinfectant action of the formaldehyde. The curves in Figure 1 were smooth in the absence of peptone, but it was not possible to draw smooth curves when this was present. The flattening of the curve could not be explained by changes in the bacterial population. Dr. Rao considered that the formaldehyde became irreversibly bound and could not be recovered even after boiling with sulphuric acid. Dr. Rao's theory was that the viable count decreased until the available formaldehyde dropped below a certain concentration, then the flat part of the curve was obtained until the spores germinated and these were killed again. Although the difference in A, B and C may have been due to the processes of manufacture, it still might well affect the uptake of formaldehyde. The reactions of formaldehyde were complex and it could react with the SH group. Vapour phase titration had not been tried. The control of pH was extremely important. Dr. Rao had said that the amide group was known to react with formaldehyde.

THE STABILITY OF SOLUTIONS OF 5-HYDROXYTRYPTOPHAN

By J. W. Hadgraft, Shirley A. P. Price and G. B. West

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Received May 15, 1958

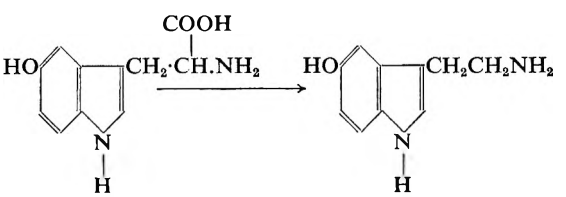
The stability of solutions of 5-HTP contained in ampoules has been studied using physical, chemical and biological methods. No loss of activity occurs when such solutions are sterilised by autoclaving provided the air in the ampoule is replaced by nitrogen. To prevent any formation of colour it is preferable to include 0·1 per cent sodium metabisulphite in the solution.

Recent investigations into the biochemistry of 5-hydroxytryptamine (5-HT) have provided evidence that this amine is formed in the body by decarboxylation of 5-hydroxytryptophan (5-HTP). Since various ataractic drugs mobilise 5-HT from the brain and other organs, the administration of 5-HT should conteract this loss, provided that it can penetrate to the sites where 5-HT normally resides and be stored there. However, it has been shown recently that 5-HT is incapable of readily penetrating the blood-brain barrier. This difficulty may be overcome by administering the amino acid precursor of the amine, as the brain possesses some 5-HTP decarboxylase activity, and injections of 5-HTP have raised the level of 5-HT in the brain and other organs. There is reason to suppose that 5-HT is related to the mechanism of nervous depression, and experimental work along these lines is in progress. Studies on the stability of solutions of this amino acid are therefore necessary.

In the present work, solutions have been maintained at a high temperature for short periods of time, since it is necessary to keep them for many months, or even years, to obtain considerable destruction at low temperatures.

Methods and results

This investigation into the stability of solutions of 5-HTP was made in two parts. In the first, the physical chemical properties of the amino acid were used; in the second, the amino acid was decarboxylated (I into II) and the resulting amine (5-HT) then tested biologically.



(I) 5-HTP (II) 5-HT

Spectrophotometric Estimation of 5-Hydroxytryptophan

The activity of the 5-HTP remaining in solution after heat treatment was determined by spectrophotometry. The material was diluted with 0·01N HCl to a strength of 25 μg./ml. and the ultra-violet absorption measured at 275·5 mμ, E (1 per cent, 1 cm.) = 264 and 295 mμ, E (1 per cent, 1 cm.) = 214[1]. Unheated solutions were similarly diluted with acid and served as controls (100 per cent).

To determine if any other indole compound was formed from 5-HTP by the heat treatment, two-dimensional chromatography was used. Solutions containing the equivalent of 5 μg. of the amino acid were applied to Whatman No. 1 paper and chromatograms were run in a glass tank by the ascending technique first with a solvent of *iso*propanol-ammonia-water (20:1:2) for 16 hours and secondly with *n*-butanol-acetic acid-water (12:3:5) for 6 hours[2]. The chromatograms were developed by dipping the paper in a tank containing an acetone solution of Ehrlich's reagent (dimethylaminobenzaldehyde). The R_F values for 5-HTP in the solvents are *iso*propanol-ammonia-water, 0·17 and *n*-butanol-acetic acid-water, 0·15.

The following solutions each containing 0·5 per cent (w/v) of DL-5-HTP were prepared and transferred to 2 ml. clear glass ampoules which were filled: (1) solution in distilled water; (2) solution in distilled water containing 0·1 per cent sodium metabisulphite; (3) solution in acetate buffer (pH 3·6); (4) solution in oxygen-free distilled water, filled in an atmosphere of nitrogen; (5) solution in oxygen-free acetate buffer (pH 3·6), filled in an atmosphere of nitrogen. All ampoules complied with the test of the British Pharmacopoeia for alkalinity of glass. The oxygen-free distilled water was prepared by boiling the water for 5 minutes in the presence of a stream of nitrogen. After dissolving the 5-HTP, nitrogen was passed through solutions (4) and (5) and also into each individual ampoule before sealing. A few ampoules in each group were kept unheated. The remainder were heated in an autoclave at 115° for 30 minutes. Some of these heated ampoules were then stored in the absence of light for 21 days at 45°. The colour and pH values of the solutions were noted and their ultra-violet absorption measured.

Solutions prepared either in water or in acetate buffer in the presence of air were the only two to show a slight colouration on autoclaving and a slight loss of activity (see Table I). On autoclaving and storage at 45°,

TABLE I

THE EFFECT OF AUTOCLAVING OR AUTOCLAVING AND STORAGE AT 45° FOR 21 DAYS ON THE COLOUR, pH AND ULTRA-VIOLET ABSORPTION (MEASURED AT 275 mμ) OF SOLUTIONS OF 5-HTP. ABSORPTION VALUE IS RECORDED AS A PERCENTAGE OF THAT OF UNHEATED SOLUTIONS.

Solvent	Autoclaved			Autoclaved and stored	
	Colour	pH	275·5 mμ	Colour	275·5 mμ
Water	Light brown	6·8	97	Dark brown	99
Water + metabisulphite	Trace	4·5	101	Light brown	97
Acetate buffer	Light brown	3·6	98	Brown	99
Water (under nitrogen)	Colourless	7·2	101	Trace	100
Acetate buffer (under nitrogen)	Colourless	3·6	100	Trace	101

Similar values for activity were obtained when absorption was measured at 295 mμ.

these two solutions darkened in colour. The solution containing 0·1 per cent of metabisulphite developed a trace of colour on storage and also showed a slight loss of activity. The oxygen-free solutions were colourless after autoclaving but developed a trace of colour on storage. The pH of the solution did not appear to have any significant effect on the stability of 5–HTP. Even the darkest solution on chromatography showed only one spot characteristic of 5-HTP. It was concluded that solutions of 5-HTP may be sterilised by autoclaving without loss of activity if the containers (ampoules) are sealed under nitrogen or contain sodium metabisulphite.

Some ampoules containing sodium metabisulphite as well as being filled under nitrogen showed no sign of deterioration after storage at room temperature for six months, whereas a black precipitate formed in control ampoules filled in air and stored under similar conditions. From these experiments, it appears that sodium metabisulphite (0·1 per cent) only partially inhibits the oxidation of 5-HTP, but is of value to prevent the slight discolouration which occurs when solutions filled under nitrogen are stored for prolonged periods of time.

Biological determination of 5-Hydroxytryptophan

The activity of the 5-HTP remaining in solution after heat treatment was determined by decarboxylating the amino acid and testing the resulting 5-HT on the rat uterus. The 5-HTP decarboxylase was prepared by grinding fresh rat kidneys in a mortar with a little sand and M/15 phosphate buffer (pH 8·0, 1 g. tissue/2 ml. buffer), allowing the mixture to stand and then pipetting off aliquots of the homogenate into specimen tubes which served as the reaction vessels. The reaction mixture in these tubes consisted of rat kidney homogenate (0·2 — 1·6 ml.), the co-enzyme, pyridoxal phosphate (100 μg.), iproniazid, an inhibitor of mono-amine oxidase, (100 μg.), DL-5-HTP (0·05–0·4 ml., 10^{-3}), and M/15 phosphate buffer (pH 8·0) to 5 ml. The substrate was added last and after mixing the solutions incubation was allowed to proceed at 37° for varying periods of time. The mixture was then cooled and the pH adjusted to 5·0 to retard further decarboxylation.

The reaction is specific as no 5-HT activity was detected after incubation in solutions containing boiled enzyme and none was present before incubation. The yield of 5-HT was not increased by using more pyridoxal or iproniazid, and when iproniazid was omitted no 5-HT activity was detected after incubation, the 5-HT being deaminated immediately on formation by the amine oxidase in the enzyme homogenate.

Bioassays were made on the isolated atropinised uterus of the rat in oestrus, according to the method of Parratt and West[3]. Usually a 2×2 design was employed. On occasion, the specificity of the response was checked using the potent anti-5-HT drug, 2-bromlysergic acid diethylamide.

To determine the optimal time of incubation, several experiments were performed using varying periods of time and substrate concentrations. Figure 1 shows the results of experiments with 100 and 800 mg. of kidney

homogenate and 50 μg. of 5-HTP, and similar types of graphs have been obtained with 400 μg. of substrate. The enzyme is a rapidly functioning one and detectable amounts of 5-HT were found within 1 minute after the addition of substrate to appropriate amounts of enzyme. It is clear that 800 mg. of kidney homogenate produced almost maximal conversion of 50 μg. of 5-HTP in 60 minutes, and in all subsequent work solutions were incubated at 37° for 60 minutes.

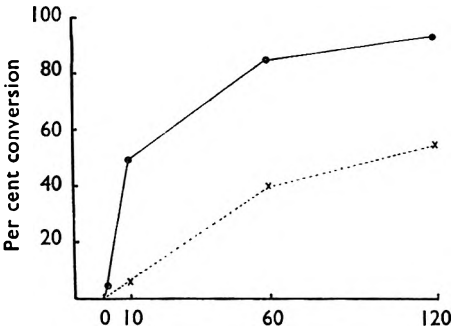


FIG. 1. The effect of varying times of incubation at 37° on the percentage of 5-HTP (50 μg.) converted into 5-HT by 100 m.g. x - - - x or 800 mg. ●—● of rat kidney.

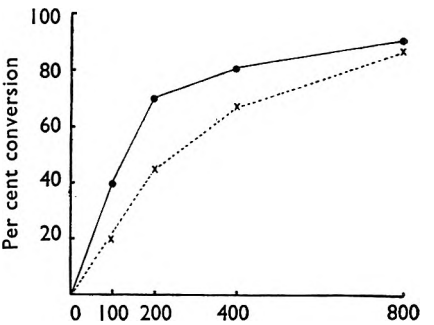


FIG. 2. The relationship between the concentration of 5-HTP decarboxylase activity of rat kidney and the percentage of 5-HTP (50 μg. ●—● or 400 μg. x - - - x) converted into 5-HT. Incubation time is 60 minutes.

The relation between the enzyme content and the concentration of substrate is shown in Figure 2. It appears that 800 mg. of rat kidney can efficiently decarboxylate 400 μg. of substrate in 60 minutes, and in the subsequent assay work the amount of tissue remained at 800 mg. but the substrate content was reduced to 50 μg.

The following solutions each containing 0·5 per cent (w/v) of DL-5-HTP were prepared and transferred to 1 ml. ampoules: (1) sclution in distilled water; (2) solution in oxygen-free distilled water, filled in an atmosphere of nitrogen, as described earlier in this paper. The ampoules in the two sets were each divided into four groups—unheated and refrigerated, unheated and stored at 45° for 6 weeks, autoclaved and

TABLE II

THE EFFECT OF HEAT TREATMENT AND STORAGE AT 45° FOR SIX WEEKS ON THE COLOUR AND ACTIVITY, ESTIMATED AS 5-HT, OF SOLUTIONS OF 5-HTP CONTAINED IN AMPOULES SEALED UNDER AIR OR NITROGEN. STANDARD REFERENCE SOLUTION (100 PER CENT) IS UNHEATED FRESH SOLUTION SEALED UNDER NITROGEN

Treatment	Storage	Air		Nitrogen	
		Colour	Activity	Colour	Activity
Unheated ..	Fresh	Colourless	100	Colourless	100
Autoclaved ..	Fresh	Light brown	90	Trace	100
Unheated ..	45°	Dark brown*	52	Colourless	100
Autoclaved ..	45°	Dark brown*	22	Trace	90

* Small black precipitate also present in these ampoules.

refrigerated, and autoclaved and stored at 45° for 6 weeks. The results of the biological assays are shown in Table II. In each experiment, the standard reference solution was an unheated fresh solution sealed under nitrogen. When the ampoule was filled with air and heated (particularly when stored at 45° for 6 weeks), loss of activity was great and the solution darkened considerably. On the other hand, solutions under nitrogen showed no loss of activity and only a trace of colouration. The pH values of all these solutions were similar (7·0–7·2).

Acknowledgements. Roche Products Ltd. kindly supplied the 5-HTP, iproniazid and pyridoxal; Upjohn Ltd. the 5-HT, and Sandoz Ltd. the bromlysergic acid diethylamide.

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STUDIES ON 5-HYDROXYTRYPTAMINE AND 5-HYDROXYTRYPTOPHAN

By G. B. West

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

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The 5-HT content and the 5-HTP decarboxylase activity of several tissues of seven mammalian species has been estimated. Whereas 5-HT is concentrated in the spleen and gastrointestinal tract, the highest enzyme activity is found in the kidney, liver, gut and brain. In rats, treatment with reserpine or chlorpromazine lowers the 5-HT content of many tissues and reduces the 5-HTP decarboxylase activity of the kidney, but prolonged treatment with cortisone only lowers the 5-HT content of the skin.

During recent years, the discovery of new facts about 5-hydroxytryptamine (5-HT) has proceeded with astonishing speed. Its function in the body however remains uncertain, though several possibilities exist—(*a*) it may play a role in controlling the activity of the central nervous system, (*b*) it may act as the stimulus to peristalsis in the gut, (*c*) it may be the factor controlling capillary permeability in the tissues, or (*d*) it may be an important haemostatic agent. Knowledge of its distribution in the tissues[1,2] may give an indication of the part it is likely to play in the normal functioning of the body, but knowledge of its formation from 5-hydroxytryptophan (5-HTP) and its destruction is equally important. Preliminary studies on the biosynthesis of 5-HT have already been reported by Gaddum and Giarman[3] and the present work extends these observations and includes the effects of two ataractic drugs as well as cortisone on the distribution and formation of 5-HT. It was hoped that such studies might shed further light on the physiological function of this amine.

Methods

Preparation of Tissues for Determining their 5-HT Content

All tissues were freshly excised, cleaned and weighed in the wet state. The desired amount of tissue was cut into small pieces and extracted with acetone (5 ml./g. tissue) for 24 hours. After decanting the acetone, the tissues were re-extracted with a similar volume of 80 per cent (v/v) acetone. The acetone from the combined filtrates was removed by evaporation in air below 30°. The residue was brought to the desired volume (1–10 ml./g. of original material) with 0·9 per cent (w/v) NaCl solution and its activity measured. Each value in Table I represents the mean of four results.

Preparation of Tissues for Determining their Content of 5-HTP Decarboxylase

Similar portions of tissues were weighed, cut into small pieces, and ground in a mortar with a little sand and M/15 phosphate buffer (2 ml./g. tissue) at pH 8·0. Such treatment extracts the enzyme but not the 5-HT.

Aliquots of the homogenates containing the desired quantity of tissue (800 mg.) were measured into specimen tubes containing the co-enzyme, pyridoxal phosphate (100 μg.), and an inhibitor of mono-amine oxidase, iproniazid (100 μg.), to prevent the destruction of the 5-HT formed from 5-HTP. Phosphate buffer was then added to bring the volume to 4·6 ml., and the substrate, 5-HTP, was added last (400 μg. contained in 0·4 ml. water). Immediately after its addition, the mixture was shaken and incubated at 37° for 1 hour (as suggested by Gaddum and Giarman[3]). The reaction was then stopped by reducing the pH of the solution to 5·0 with N HCl, and the 5-HT content of the solution assayed. The amount of 5-HT formed per gram of tissue may be used as an indication of the 5-HTP decarboxylase content of that tissue. Each value in Table II represents the mean of four results.

Bioassay Procedure

Bioassays were carried out on the isolated uterus of the oestrous rat. An aerated 15-ml. bath of de Jalon's fluid containing atropine (10^{-7}) at 28° was used. On occasion, the extracts were also assayed on the rat colon suspended in a similar bath at 20°. Usually, both preparations were sensitive to 0·01–0·02 μg. 5-HT (i.e., approximately 10^{-9} g.). The specificity of the reaction was checked by using the 5-HT antagonist, 2-bromlysergic acid diethylamide. The standard 5-HT was used as its creatinine sulphate, but values given in the text refer to the base.

Depletion of 5-HT Stores in the Rat

Two groups of 10 female albino rats (100–150 g. in weight) received an intraperitoneal injection of either reserpine (1 mg./kg.) or chlorpromazine (25 mg./kg.) on each of 3 consecutive days. A third group of rats were given daily an intramuscular dose of cortisone (50 mg./kg.) for 14 days, whilst a fourth control group received a daily intraperitoneal dose of normal saline (0·5 ml.) for 14 days. All animals were killed 24 hours after the last injection and several tissues taken for 5-HT assay. The 5-HTP decarboxylase activity of the kidneys of rats from each group was also measured.

Results

Tissue distribution of 5-HT

The results are shown in Table I. Briefly, 5-HT is concentrated in the spleen and gut of all the species studied. Another important site in the rat

TABLE I

5-HT CONTENT (μG./G.) OF ANIMAL TISSUES

Tissue	Rat	Mouse	G'pig	Hamster	Rabbit	Dog	Cat
Kidney	0·1	0·1	0·1	0·1	0·1	0·1	0·1
Liver	0·1	0·7	0·1	0·2	0·6	0·5	0·6
Spleen	2·5	2·7	1·1	20·5	24·3	4·6	8·5
Skin	1·3	0·4	0·1	0·1	0·1	0·1	0·1
Stomach	1·4	1·0	1·4	1·2	4·9	5·2	0·5
Duodenum ..	1·2	1·2	5·0	0·9	3·3	3·7	0·9
Ileum	1·2	1·0	3·4	1·3	3·7	4·3	0·5
Brain	0·2	0·3	0·3	0·2	0·3	0·2	0·3

is the skin where over half of the 5-HT content of the body is located[2]. Only traces of 5-HT are found in the kidney and brain.

Tissue Distribution of 5-HTP Decarboxylase

The enzyme activities of the tissue preparations are recorded in Table II. By far the most striking result is that the highest concentrations of enzyme are found in the kidney and liver, two tissues where the 5-HT content is low. The 5-HT-forming capactity of the spleen, however, is

TABLE II

THE RELATIVE 5-HTP DECARBOXYLASE ACTIVITY OF VARIOUS ANIMAL TISSUES, RECORDED AS THE 5-HT FORMED (μG./G. TISSUE) FROM ADDED 5-HTP

Tissue	Rat	Mouse	G'pig	Hamster	Rabbit	Dog	Cat
Kidney	188	187	171	62	56	47	24
Liver	125	22	156	62	94	12	55
Spleen	1	6	2	93	19	2	2
Skin	1	1	1	1	1	1	1
Stomach ..	1	2	64	45	34	3	25
Duodenum ..	2	2	391	9	13	2	23
Ileum	1	1	190	1	6	1	8
Brain	32	9	13	20	5	2	2

small, except in the hamster and rabbit, and the skin likewise is deficient in this enzyme. Only traces of enzyme activity are found in the gut of the rat, mouse and dog, but exceptionally high values exist in this region in the guinea pig. The brain of several species is also capable of forming much 5-HT.

Action of Reserpine in the Rat

After reserpine treatment, the 5-HT content of several tissues was reduced to less than 10 per cent of the control values of untreated animals (Table III). The gut on the other hand lost only about 50 per cent of its content. The 5-HTP decarboxylase activity of the kidneys of these rats was reduced to 48 per cent of that of saline-injected rats. This reduction in enzyme activity was due in part to the residual reserpine, since reserpine (10^{-5}) added to homogenates of normal rat kidney always slightly reduced the conversion rate of 5-HTP to 5-HT.

Action of Chlorpromazine in the Rat

Chlorpromazine lowered the 5-HT content of the tissue studied though it was less active than reserpine (Table III). Similarly the 5-HTP

TABLE III

THE 5-HT CONTENT OF TISSUES OF THE RAT AFTER DRUG TREATMENT. ALL VALUES ARE EXPRESSED AS PERCENTAGES OF THOSE OF UNTREATED ANIMALS

Tissue	Reserpine	Chlorpromazine	Cortisone
Spleen ..	5	25	100
Skin	7	17	35
Stomach ..	50	70	110
Duodenum ..	45	50	100
Ileum	70	90	109
Brain	9	40	90

decarboxylase activity of the kidneys of the treated rats was reduced, this time to 64 per cent of that of the saline-treated animals.

Action of Cortisone in the Rat

The action of cortisone on the 5-HT levels in the tissues differed from that of reserpine or chlorpromazine. Only the skin showed a major change, being reduced to 35 per cent of that of the control level (Table III) There was no alteration in the 5-HTP decarboxylase activity of the kidney.

Discussion

The finding that 5-HT is present in extracts of brain aroused considerable speculation a few years ago concerning its function in the central nervous system. Hallucinogens were thought to exert their effect by antagonising the brain 5-HT content and the hypothesis was advanced that the 5-HT content of the brain is one of the factors controlling the activity of nerve cells in the brain. Recent work however has indicated that this is not the full explanation and the role of 5-HT in the brain is still open. In the present experiments, the 5-HT-forming enzyme has been detected in the brain of all species studied, and it is particularly active in the rat, hamster and guinea pig.

The present results also show that there is generally little or no relation between the 5-hydroxytryptophan decarboxylase activity of a tissue and the amount of 5-HT that can be extracted from that tissue. For example, the decarboxylase activity of the kidney and liver is high yet their 5-HT content is low. In these two tissues, this can probably be explained by the high activity of mono-amine oxidase, an enzyme which rapidly inactivates the 5-HT. In contrast, the spleen of most species contains much 5-HT but little enzyme. Both the decarboxylating enzyme and 5-HT have been found in high concentration in the stomach, duodenum and ileum of most species, and this is in accord with Erspamer's view that the gut is one of the most important sites of 5-HT production.

The hypothesis has been advanced that reserpine owes its pharmacological properties to its ability to interfere with the binding sites of 5-HT in the body[4]. Reserpine mobilizes the 5-HT from the brain, blood platelets and gastrointestinal tract and it is now apparent that the 5-hydroxytryptophan decarboxylase activity of the kidney of the rat can be reduced by treatment with reserpine. This result is in sharp contrast to that of Brodie and his colleagues[4] who found that this drug did not modify the decarboxylase activity of the brain of the rabbit. Chlorpromazine also reduces the 5-HT content of the brain, spleen and skin and lowers the decarboxylase activity of the kidney. Cortisone however reduces the 5-HT content of only the skin without modifying the kidney decarboxylase activity. Thus it is clear that much work still remains to be done on the action of drugs on the biosynthesis and metabolism of 5-HT.

The 5-hydroxytryptophan decarboxylase activity of a particular tissue has been determined as the amount of 5-HT formed in 60 minutes by 1 g. of tissue. It is possible that different conclusions might have been reached if the decarboxylation had been allowed to proceed for longer

periods of time than 1 hour or if the amount of substance in the system was altered.

Acknowledgements.—I wish to express my thanks to Roche Products, Ltd. for the supply of iproniazid and pyridoxal; Ciba Laboratories Ltd. for reserpine; Upjohn Ltd. for 5-HT; May and Baker Ltd. for chlorpromazine; and Sandoz Ltd. for 2-bromlysergic acid diethylamide.

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DISCUSSION

The papers were presented by MISS SHIRLEY A. P. PRICE and DR. G. B. WEST.

The CHAIRMAN. Could more details be given of the technique of filling the ampoules in an atmosphere of nitrogen, and had the oxygen content been determined in any filled ampoules?

DR. G. F. SOMERS (Liverpool). How did one associate the presence of a substance like 5-hydroxytryptamine in a particular tissue with its function?

DR. F. HARTLEY (London). Was Dr. West satisfied with the specificity of the decarboxylases he had determined, and would the extraction process used for 5-HT deal with bound as well as free 5-HT?

MR. J. J. LEWIS (Glasgow). The action of reserpine appears to be non-specific. For example, in the brain it reduces not only the 5-HT content of a tissue but also its noradrenaline content.

MR. S. G. E. STEVENS (London). What were the decomposition products in the solutions? Had the authors looked for 3:5-dihydroxyindole, which could arise from 5-HTP?

MR. C. A. JOHNSON (Nottingham). The ultra-violet figures in Table I (p. *88 T*) indicated a full content of indole even in coloured solutions; this throws doubt on the chromatographic system used.

MR. H. B. HEATH (Sudbury). Was the loss of 50 per cent in the biological activity sudden and were solutions examined at periods between 3 and 6 weeks?

DR. WEST replied. The presence of 5-HT in a tissue is not indicative of its site of action since its concentration depends upon the activities of the enzymes responsible for its formation and its destruction. For example, the 5-HT content of the brain can easily be increased by an inhibitor of the inactivating enzyme. The homogenates of liver had been made in the usual way and contained all the enzymes originally present, yet incubation of these homogenates with several amino acids other than

5-hydroxytryptophan failed to yield detectable amounts of the corresponding amines. This indicated the specificity of the reaction when carried out with the reported method. The extraction process used dealt with free 5-HT. Bound 5-HT is extracted only with difficulty.

MISS PRICE also replied. Some solutions 10 months old filled under nitrogen with 0·1 per cent sodium metabisulphite were water-white. She was uncertain about the nature of the brown compound, though it was probably a melanin derivative. The solutions had been boiled for 20 minutes and then cooled whilst nitrogen was bubbled through and the ampoules were then filled under nitrogen. The solutions were not examined at periods between 3 and 6 weeks. The coloured solution had never shown any indole derivative other than 5-HTP by two-dimensional chromatography in several solvent systems.

THE PRECISION OF SOME PROCEDURES IN PHARMACEUTICAL ANALYSIS

PART I. USE OF A PIPETTE AND A BURETTE

BY A. R. ROGERS

From the School of Pharmacy, Brighton Technical College

Received May 22, 1958

Estimates have been made of the variance associated with the calibration of a number of 10- and 20-ml. pipettes and 50-ml. burettes of Grade B quality, and of the variance associated with their use by a class of students. Comparison with the variance of the results of students performing simple titrations with this apparatus indicates that the chief components of the latter variance have been identified.

THE tolerances for the purity of official drugs are framed to take into account variations due to the sampling and assay procedure used, as well as variations due to manufacturing processes. Saunders and Fleming[1] have pointed out that "it would be extremely useful if the percentage standard deviation of the different assay methods of the British Pharmacopoeia could be published in the monographs. The data for calculating them is available in the schools of pharmacy and probably also in a number of industrial analytical laboratories".

During the last few years, a number of workers[2–4] have studied the precision and accuracy of the weighing and measuring operations of extemporaneous dispensing. In those experiments where the accuracy of dispensing is checked by a physical or chemical assay, it is necessary to establish that the assay errors are insignificant in relation to the variations in the dispensing. Although this should always be established within the experimental pattern of the dispensing measurements, it would be helpful in designing and planning these experiments if an "external" estimate of the precision and accuracy were already available.

A detailed discussion of errors associated with the use of volumetric apparatus has been given by Conway[5]. In this paper, an attempt is made to estimate the variation associated with the use of a pipette and a burette by students. The appropriate sum of the variances of these individual operations will be an underestimate of the variance of a complete titration, because not all of the sources of variation will have been identified and measured. The total variance of a titration, estimated by this "synthetic" process, should therefore be compared with the variance of the results obtained in practice. This approach is similar to that adopted by Capper and Dare[4] in an investigation of the precision of measuring and weighing operations in dispensing.

It is important to note that it is not the accuracy (or "correctness") but the precision or reproducibility of analytical operations that is being studied in this series of papers. Nevertheless, since each member of the class of students used a different pipette and burette in the experiments where a complete titration was performed, the accuracy of calibration of

these pieces of apparatus was checked so that appropriate allowance could be made for this source of variation.

Use of a Pipette

About 80 students and members of staff of the school were instructed to proceed according to the following method[6], on one occasion with a 10-ml. pipette, on another with a 20-ml. pipette. Fill the pipette by suction from the vessel, which contains distilled water at 21·0° ±0·5°, to about 2 cm. above the mark. Close the upper end of the pipette with the tip of the dry finger, and wipe any adhering water from the outside of the lower stem. Allow the water to run out slowly by slightly relaxing the pressure of the finger. Hold the pipette vertically so that the mark is at the same level as the eye, and tighten the finger on the mark when the meniscus just reaches the graduation mark. Remove any drops adhering to the tip by stroking against a glass surface. Allow the water to run out into the (already tared) weighing bottle, the tip of the pipette touching the wall of the bottle. When the continuous discharge has ceased, hold the jet in contact with the side of the bottle for a further 15 seconds. Then remove the pipette from contact with the bottle, thus removing any drop adhering to the outside of the pipette.

The weighing bottle was weighed to the nearest 0·1 mg. on a Sartorius "Selecta" semi-micro balance by the author before and after the delivery of the water, and the volume of water which had been delivered was calculated. All weighings were performed within 3 hours of the pipetting, and the bottles were kept stoppered except while being used. The same 10- or 20-ml. pipette was used by every worker taking part in the experiment. The pipettes complied with British Standard, 1583 : 1950.

The standard deviation of the volumes was calculated after rejection of those results, about 10 per cent of the total, where it was observed that the person using the pipette had either not allowed 15 seconds for after-drainage, or not held the jet of the pipette against the side of the bottle during the drainage period, or both.

As a check of this method of estimating the standard deviation, a straight line was drawn by eye through those points on a plot of probit of cumulative frequency against volume of water that came within the range of probits four to six, although attention was also paid to the trend of points lying outside thise range; the reciprocal of the slope of this line was taken[7] as an estimate of the standard deviations of those volumes that had been pipetted according to the established procedure. Tests for "outliers" in observational data have been described[8], but in this procedure the suspected "outliers" were not rejected outright; instead, less weight was attached to them than to the rest of the results. Almost identical standard deviations resulted from the application of these two different procedures, namely 0·0092 ml. for a 10-ml. pipette and 0·0204 ml. for a 20-ml. pipette. The contribution towards the total variance of a titration made by the use of a pipette, assumed to be correctly calibrated, was therefore taken as 0·00008 ml.^{2} for a 10-ml. pipette and 0·00042 ml.^{2} for a 20-ml. pipette.

The raw data are plotted sequentially in Figures 1 and 2. In each diagram, the continuous horizontal line represents the best estimate of the mean, and the dotted lines represent one standard deviation above and below the mean, respectively.

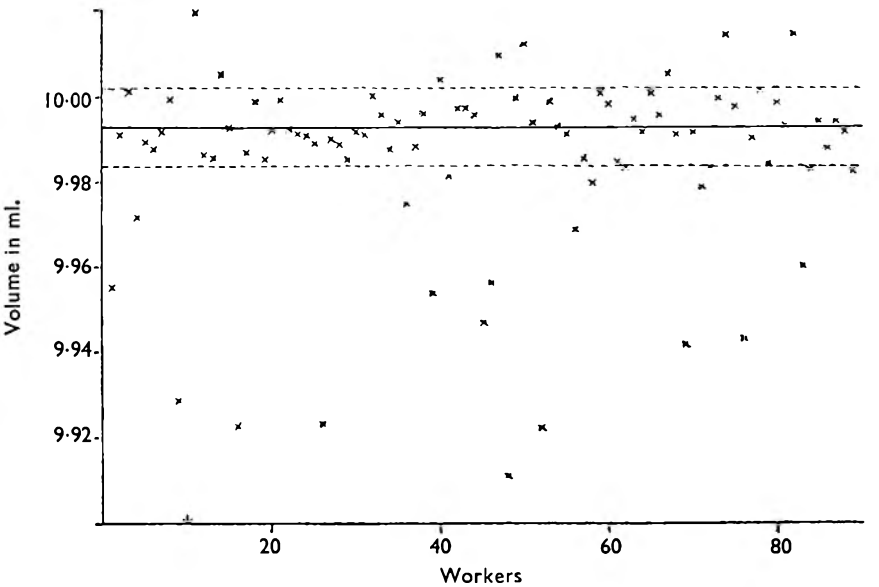

FIG. 1. Volumes of water delivered by different workers using the same 10-ml. pipette. —— Mean. - - - - One standard deviation above and below the mean.

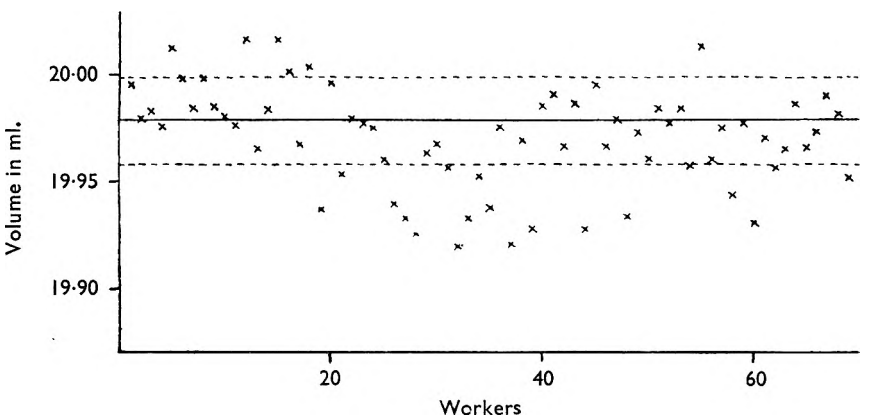

FIG. 2. Volumes of water delivered by different workers using the same 20-ml. pipette. —— Mean. - - - - One standard deviation above and below the mean.

USE OF A BURETTE

About 90 students and members of staff were instructed to read six burettes and record the level of the liquid in each. The burettes were all sealed, top and bottom, and one contained a thermometer which

showed that the temperature of the contents stayed at 21·0° ±0·5° while the experiment was in progress. Four burettes contained distilled water and two contained 0·1N aqueous potassium permanganate. The liquid levels were arbitrary, around the 5-ml. mark in all cases.

In a small number of instances, less than 1 per cent of the total, a gross mistake in reading the burette occurred, for example 5·93 ml. was recorded instead of 4·93 ml.; in these the recorded figure was corrected. No corrections of this type were made unless the correction was exactly 1·00 ml. or, in one reading, 0·50 ml. Mistakes less than 0·50 ml. in magnitude, if they occurred, were included and may have contributed towards the total variance.

For each person collaborating, the average of the readings of the four burettes with water was calculated. A histogram of the distribution of these averages showed that there were two peaks to the frequency distribution (see Fig. 3), which were separated by about 0·04 ml. These are

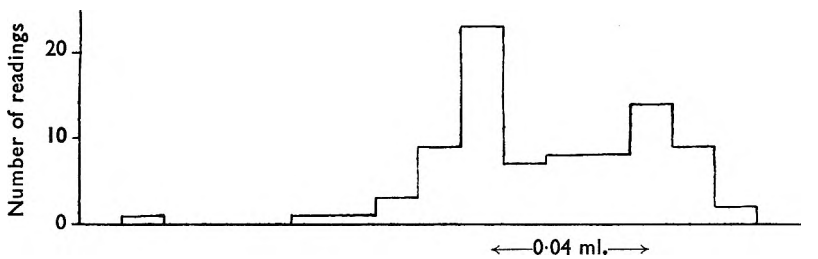

FIG. 3. Histogram showing frequency distribution of average burette readings with water.

presumably caused by some workers observing the "true" meniscus and others the "false" meniscus[2]. For aqueous potassium permanganate, the histograms of the average burette readings showed two quite distinct peaks separated by about 0·15 ml. (see Fig. 4). It is thought that these are caused by some workers observing the top of the meniscus and others the bottom of the meniscus.

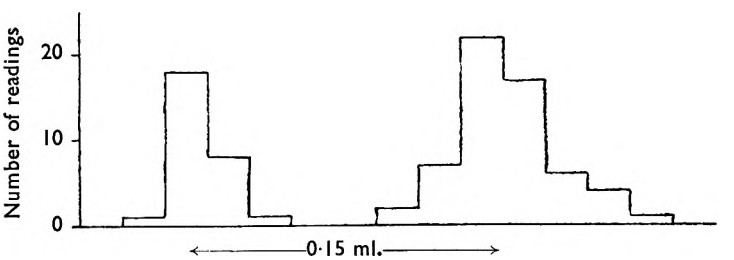

FIG. 4. Histogram showing frequency distribution of average burette readings with 0·1 N aqueous potassium permanganate.

It was clear from the raw data that workers were almost without exception consistent in reading either the top or the bottom of the meniscus, or the "true" or the "false" meniscus. This is important when it is remembered that in practice a burette reading is one of a pair of

readings, and it is the difference between the pair of readings that must be known accurately. Mechanical aids to reading a burette are quite commonly used in practice, and this also would help to ensure that the meniscus is observed in a consistent manner.

The six sets of burette readings were taken in pairs, namely two pairs for water and one pair for aqueous potassium permanganate. For each pair, the probit of the cumulative frequency was plotted against the value of the difference between the two burette readings. The standard deviation of the difference between a pair of burette readings was estimated from the slope of a line through the points in the manner described above. The results for water were 0·0264 ml. and 0·0238 ml.; these gave an F-value of 1·234 with 86 and 85 degrees of freedom, so the estimates were pooled to give a standard deviation of 0·0252 ml. for water. The result for aqueous potassium permanganate was a standard deviation of 0·0314 ml.

The raw data showed that in almost all cases the burettes had been read to the nearest 0·03 ml. Since the true distribution of frequency was continuous, and the frequency tapered off to zero in both directions, the variance calculated from the discontinuous data was corrected for the grouping effect by subtraction of one-twelfth of the square of the class-interval, 0·03 ml. (Sheppard's correction[9]). The contribution towards the total variance of a titration made by reading the burette, assumed to be correctly calibrated, before and after the titration was therefore taken as 0·00056 ml.^{2} for transparent aqueous solutions and 0·00091 ml.^{2} for aqueous potassium permanganate.

Calibration of Pipettes

The calibration of the forty-one 10-ml. pipettes used by the class of students were checked by the author by the procedure described under "use of a pipette". All were of grade B quality (as labelled by the manufacturers) or better, in calibration. In addition, the volume of one pipette was determined 29 times in replicate, so as to obtain an estimate of the variance of the calibration procedure; this was found to be 0·00003 ml.^{2}

The apparent variance of the forty-one 10-ml. pipettes was 0·00034 ml.^{2}, so the corrected estimate of the contribution towards the total variance of the titrations of a class of students made by faulty calibration of the 10-ml. pipettes was taken as 0·00031 ml.^{2}, corresponding to a standard deviation of 0·018 ml.

Similar experiments were made on the 20-ml. pipettes. The apparent variance of the 20-ml. pipettes was 0·00086 ml.^{2} and the variance of the calibration procedure was again 0·00003 ml.^{2}, so the corrected estimate of the contribution towards the total variance of the titrations of a class of students made by faulty calibration of the 20-ml. pipettes was taken as 0·00083 ml.^{2}, corresponding to a standard deviation of 0·029 ml.

It is appreciated that the figures reported in this section are of little interest outside the context of this paper. The calibration of a pipette

is a simple and rapid matter, and it would be expected that analysts in general use pipettes of Grade A quality or better, so that the usual variance from this source may be less than reported here.

Calibration of Burettes

A small random sample of burettes used by the class of students was taken, and the calibrations checked at 2-ml. intervals by the author according to the following procedure[6].

Fill the burette with water to a short distance above the zero mark, and slowly run out water until the meniscus is exactly on the zero mark. Remove the drop of water adhering to the jet by bringing the jet into contact with a glass surface. Allow the burette to discharge freely into the (already tared) bottle. When the meniscus of the water is about 1 cm. from the line to be tested, reduce the rate of outflow so that the motion of the water surface is brought under complete control, and adjust the meniscus exactly on the mark. Remove the drop adhering to the jet after the setting has been made by bringing the side of the bottle into contact with the jet.

In each instance the discharged water was weighed as described before. The volumes were calculated and the discrepancies between the observed and the theoretical volumes and hence the variance of the discrepancies were calculated. In addition, the variation of the calibration procedure was estimated by replicate determinations to be 0·00002 ml.^{2} The variances of the errors of the graduations of the seven burettes studied, corrected for the calibration procedure variance, ranged from 0·00023 to 0·00061 ml.^{2} The application of Bartlett's test[10] showed the absence of heterogeneity of the various estimates so they were pooled to give a variance of 0·00039 ml.^{2}

Because a burette is read twice in a complete titration, the corrected estimate of the contribution to the total variance of the titration results of the class of students made by faulty calibration of the burettes was taken as $2 \times 0{\cdot}00039 = 0{\cdot}00078$ ml.^{2}, corresponding to a standard deviation of 0·028 ml. The estimate is based on rather a small sample of the 41 burettes used by the students, but it is thought not to be seriously in error. All of the burettes tested were found to be of grade B quality, as labelled by the manufacturers, in calibration, and apart from one burette they were only just outside the grade A tolerances. This is a fortunate occurrence in view of the length of time required to check the markings of a burette.

Discussion

Records have been kept during the present session of all classwork in quantitative analysis performed by the 40 or so first-year degree and diploma students whose pipettes and burettes had been checked. It is hoped in Part II of this series of papers to present a detailed report of the reproducibility of their results in titrations, and to discuss the value of the results in estimating the relative standard deviations of the official

assay procedures. Here it is sufficient to say that in the easiest and most accurate titrations, such as peroxide—permanganate and some acid—base titrations, which involve one pipetting operation and the use of a burette, a coefficient of variation (relative standard deviation) of results of about 0·25 per cent was found.

Taking the variances for the operations to be those given earlier in this paper, the coefficient of variation of results in a titration where 20 ml. of solution, taken by pipette, gives a titre of 20 ml. of a colourless reagent would be,

$$\sqrt{\frac{0{\cdot}00042 + 0{\cdot}00083}{20^2} + \frac{0{\cdot}00056 + 0{\cdot}00078}{20^2}} = 0{\cdot}0025 \text{ or } 0{\cdot}25 \text{ per cent.}$$

Similarly for 10 ml. of solution giving a titre of 35 ml. of 0·1N aqueous potassium permanganate, the coefficient of variation of the results would be,

$$\sqrt{\frac{0{\cdot}00008 + 0{\cdot}00031}{10^2} + \frac{0{\cdot}00091 + 0{\cdot}00078}{35^2}} = 0{\cdot}0023 \text{ or } 0{\cdot}23 \text{ per cent.}$$

These figures are of the same order of magnitude as the experimental results for the titrations performed by the class of students using the apparatus in those cases where there were no special difficulties or other large sources of variation or error. The discrepancies which do exist can be attributed to (*a*) difficulty in deciding on the indicator colour to select as the end point, (*b*) temperature differences, (*c*) use of dirty apparatus, (*d*) incorrect use of pipette and burette, (*e*) irregularities in the amounts of other reagents added, (*f*) loss by splashing, and (*g*) other factors not identified. The effect of factor (*a*) will vary from one type of titration to another, but it is probably very small in the most favourable instances. For most aqueous solutions, factor (*b*) is small enough not to be significant, though correction must be made with solvents such as glacial acetic acid. Factors (*c*) to (*f*) are difficult to measure, and since they are "mistakes" that should not occur, no attempt to estimate their magnitude has been made.

It is concluded that the chief sources of variation in titrimetric results, with the exception of the "indicator blank", have been identified and measured.

Acknowledgements. The author thanks the fellow-members of staff and the students of this school who provided much of the data, and the Council of the Pharmaceutical Society for the loan of a Monroe model CAA 10–3S electric calculating machine.

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DISCUSSION

The paper was presented by the AUTHOR.

The CHAIRMAN. Moran had shown that duplicate analyses made at the same time were not truly random. Would the Author's results have been different if the readings had not been made on the same day? How was the apparatus cleaned?

DR. D. C. GARRATT (Nottingham). Would the Author present figures from industrial laboratories together with his other results in future papers?

DR. J. G. DARE (Leeds). It had been found that the standard deviation in the analysis of sulphuric acid by industrial analysts was 0·3 per cent which was close to the author's own figure for a simple titration of 0·25. He had found that when students expected to obtain the same answer in a duplicate determination they tended to do so.

MR. H. D. C. RAPSON (Betchworth). He had collected similar data and often found skew results. If statistical methods were applied, a skew parameter as well as standard deviation should be considered.

DR. L. SAUNDERS (London). He had expected a reliability of about 0·2 per cent in a volumetric analysis, a standard deviation of 0·25 per cent seemed high. Was it correct to add the figures for the calibration variants at this stage?

MR. C. A. JOHNSON (Nottingham). In his experience the greatest source of error in a volumetric determination was the recognition of the end point.

MR. G. R. WILKINSON (London). Figures obtained by a number of skilled and unskilled analysts for the factor of sulphuric acid using the same reagents and apparatus, varied from 0·998 to 1·002.

MR. ROGERS. Chromic acid was used for cleaning burettes, with 20 washings with tap water followed by 3 or 4 with distilled water. Skewness was showing up all the time, sometimes one way, sometimes the other, even in the same assay, but he thought he knew the cause. Many of the experiments were spead over three or four days. The figure of 0·25 per cent had been quoted based on unpublished work. He included calibration figures so that in a future paper he could be sure that the figures achieved using the apparatus were not markedly discrepant from their best results. He hoped that industrial firms would publish their figures.

NEUROMUSCULAR BLOCKING AGENTS

PART II. THE PREPARATION AND PROPERTIES OF A SERIES OF *NSN*- AND *NNN*-TRIS-ETHONIUM COMPOUNDS

BY D. EDWARDS, J. J. LEWIS, J. B. STENLAKE AND M. S. ZOHA*

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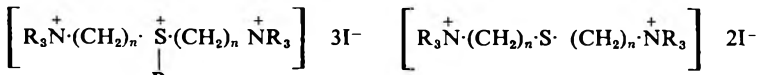
The preparation of two further *NSN*-tris-ethonium compounds, 9-ethyl-9-thioniaheptadecylenebis(triethylammonium) triiodide (dioctasulphonium triethiodide; DOSE) and 11-ethyl-11-thioniaheneicosylenebis(triethylammonium) triiodide (didecasulphonium triethiodide; DDSE) is described. The bis-quaternary compound 7-dioxothiatridecylenebis(triethylammonium iodide), and the *NNN*-tris-quaternary compounds 7:7-diethyl-7-azoniatridecylenebis (triethylammonium) triiodide (dihexazonium triethiodide; DHAE), 9:9-diethyl-9-azoniaheptadecylenebis(triethylammonium triiodide (dioctazonium triethiodide; DOAE) and 11:11-diethyl-11-azoniaheneicosylenebis(triethylammonium) triiodide (didecazonium triethiodide; DDAE) have also been synthesised. All the compounds possess neuromuscular blocking activity in the gastrocnemius muscle-sciatic nerve preparation of the cat, the phrenic nerve-diaphragm preparation of the rat and kitten and as measured by the rabbit head drop and mouse paralysis methods. Dihexazonium triethiodide and the sulphone 7-dioxathiatridecylenebis(triethylammonium iodide) (dihexone) show tubocurarine-like activity; dioctasulphonium triethiodide and dioctazonium triethiodide were predominantly tubocurarine-like but had some transitional properties, whilst didecasulphonium triethiodide and didecazonium triethiodide resembled decamethonium. Dihexazonium triethiodide was about equipotent with tubocurarine on the cat. Marked species variations in potency were observed. Theoretical implications are discussed.

CHEMICAL

IN a recent communication[1] we described the preparation and properties of two compounds, 6-ethyl-6-thioniaundecylenebis(triethylammonium) triiodide (I, $n = 5$, R = Et; DPSE) and 7-ethyl-7-thioniatridecylenebis (triethylammonium) triiodide (I, $n = 6$, R = Et; DHSE), which were found to have neuromuscular blocking activity similar to that of gallamine and tubocurarine. An attempt to prepare the compound I ($n = 4$, R = Et) from the bis-quaternary compound II ($n = 4$, R = Et) was not



(I) (II)

successful. Since it now appears that the ease with which these tris-onium salts can be formed and the neuromuscular blocking activity of onium compounds can both be related not only to the inter-nitrogen distance

* Pakistan Government Scholar.

but also in some measure to the nature of the alkyl group, the factors responsible for the failure of this reaction merit discussion at this juncture.

There can be no direct steric interference with the approach of an alkyl halide to the sulphur link. Failure to react is, therefore, the result of direct (electrostatic) interaction between the quaternary nitrogens, and the sulphur electrons, an effect which should increase rapidly as the distance separating the interacting centres decreases. The further observations[1] that the reaction of ethyl iodide with the sulphide (III, $n = 5$, R = Et) can be carried out stepwise to give first the bis-quaternary compound (II, $n = 5$, R = Et) and then the tris-onium compound (I, $n = 5$, R = Et), whilst only one product, the tris-onium compound (I, $n = 6$, R = Et), can be obtained from the sulphide (III, $n = 6$, R = Et), shows that introduction of the third onium group becomes progressively easier as the distance separating the *N*- and *S*- groups increases.

$$R_2N{\cdot}(CH_2)_n{\cdot}S{\cdot}(CH_2)_n{\cdot}NR_2 \qquad \left[Et_3\overset{+}{N}{\cdot}(CH_2)_6{\cdot}SO_2{\cdot}(CH_2)_6{\cdot}\overset{+}{N}Et_3\right] \; 2I^-$$

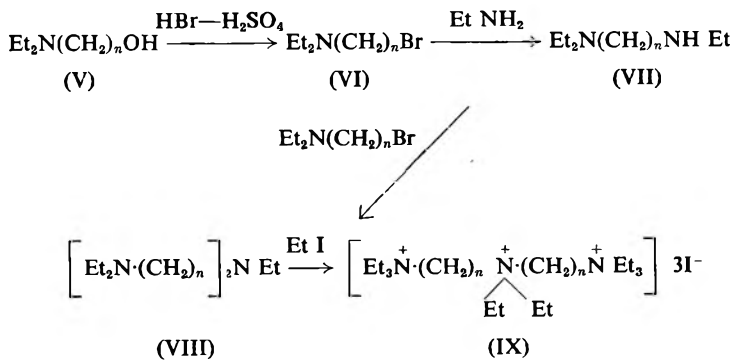
(III) (IV)

That reaction of the sulphide (III, $n = 6$, R = Me) with methyl iodide can also be brought about as a two stage reaction, with the isolation of the intermediate (II, $n = 6$, R = Me), shows that reaction at the sulphide link is also dependent on the nature of the *N*-alkyl substituent. This too can be related to the influence of the residual positive charge on the quaternary ammonium groups; an influence which would be diminished by the greater + I effect of ethyl compared to methyl substituents[2]. The possible significance of these effects in determining the level of neuromuscular blocking activity of quaternary salts will be discussed in later communications.

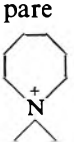
We have now prepared the longer chain *NSN*-tris-onium compounds (I, $n = 8$, R = Et) and (I, $n = 10$, R = Et) to study the effect of increasing the distance between quaternary centres on neuromuscular blocking activity in this series. 11-Ethyl-11-thioniaheneicosylenebis (triethylammonium) triiodide (I, $n = 10$, R = Et; didecasulphonium triethiodide; DDSE) was obtained by reaction of the known bis-10-diethylaminodecyl sulphide[3] with excess ethyl iodide. The octamethylene compound, 9-ethyl-9-thioniaheptadecylenebis (triethylammonium) triiodide (I, $n = 8$, R = Et; dioctasulphonium triethiodide; DOSE) was obtained similarly from bis-8-diethylaminooctyl sulphide. The latter was prepared by chain extension from 6-chlorohexyldiethylamine by the method used in the preparation of bis-5-diethylaminopentyl sulphide from 3-chloropropyldiethylamine[3]. The bis-quaternary sulphone (IV), was also prepared for comparison of its neuromuscular blocking properties with those of dihexasulphonium triethiodide (DHSE)[1]. It was obtained by treating bis-6-diethylaminohexyl sulphone[3] with ethyl iodide.

The function of the tertiary sulphur group in determining the properties of the *NSN*-tris-onium compounds has been further investigated by the

preparation and pharmacological examination of a parallel series of *NNN*-tris-quaternary compounds (IX) which were obtained by the following reaction sequence:



6-Bromohexyldiethylamine (IV, $n = 6$), obtained from 6-hydroxyhexyldiethylamine (V, $n = 6$), reacted with excess ethylamine to yield 6-ethylaminohexyldiethylamine (VII, $n = 6$) together with a small amount of bis-6-diethylaminohexylethylamine (VIII, $n = 6$). 6-Chlorohexyldiethylamine failed to react with ethylamine. Reaction of 6-bromohexyldiethylamine with 6-ethylaminohexyldiethylamine gave only poor yields of bis-6-diethylaminohexylethylamine (VIII, $n = 6$). This is due to the instability of 6-bromohexyldiethylamine, which readily cyclises on heating to yield 1:1-diethyl-1-aza*cyclo*heptylinium bromide (X) (compare with the cyclisation of 5-chloropentyldiethylamine[4]). On the other hand, 6-chlorohexyldiethylamine, although less reactive than the corresponding bromo compound, was much more stable, and could be condensed under more vigorous conditions with 6-ethylaminohexyldiethylamine to give improved yields of the base (VIII, $n = 6$). The latter, when treated with excess ethyl iodide, as before, gave 7:7-diethyl-7-azoniatridecylenebis (triethylammonium) triiodide (IX, $n = 6$; dihexazonium triethiodide; DHAE). 9:9-Diethyl-9-azoniaheptadecylenebis (triethylammonium) triiodide (IX, $n = 8$; dioctazonium triethiodide; DOAE) was obtained by an analagous series of reactions.



(X)

In the preparation of the *NNN*-decamethylene compound 10-bromodecyldiethylamine (VI, $n = 10$) was found to be sufficiently stable for it to be purified by distillation, and the required base, bis-10-diethylaminodecylethylamine (VIII, $n = 10$) was obtained by reaction of this bromo compound with 10-ethylaminodecyldiethylamine (VII, $n = 10$). Reaction of the latter with ethyl iodide as before gave 11:11-diethyl-11-azoniaheneicosylenebis (triethylammonium) triiodide (IX, $n = 10$; didecazonium triethiodide; DDAE).

Experimental

Melting points are uncorrected. We are indebted to Dr. A. C. Syme and Mr. W. McCorkindale for the microanalyses.

6-*Ethylaminohexyldiethylamine*. 6-Hydroxyhexyldiethylamine[3] (35·2 g.) in hydrobromic acid (48 per cent; 95 ml.) and sulphuric acid (33 ml.) was refluxed for 4 hours, cooled, and poured into water (1 l.). The solution was basified with sodium carbonate and extracted with chloroform. The chloroform extract was dried (Na_2SO_4) and the bulk of the chloroform evaporated under reduced pressure to yield a reddish-brown oil containing crystalline material. Excess ethylamine (40 ml.) was added to the crude 6-bromohexyldiethylamine and the mixture refluxed for 2 hours. Evaporation of the ethylamine and chloroform yielded a damp crystalline mass, which was basified and extracted with ether. Evaporation of the ether gave an oil (24·8 g.) which was distilled to yield 6-*ethylaminohexyldiethylamine*, b.p. 86–89°/0·55 mm., n_D^{17} 1·4493 (21·5 g.; 53 per cent). *Dihydrochloride* (from ethanol-ether), m.p. 172–173°. Found: C, 51·8; H, 10·6; Cl, 25·8 per cent. $C_{12}H_{30}N_2Cl_2$ requires C, 52·7; H, 11·1; Cl, 25·9 per cent.

Some of the crystalline material was filtered from the crude 6-bromohexyldiethylamine and re-crystallised from ethanol-ether to give 1:1-*diethyl*-1-*aza*cyclo*heptylinium bromide*, m.p. 250° (decomp.). Found: N, 5·9; Br, 33·9 per cent. $C_{10}H_{22}N$ Br requires N, 5·9; Br, 33·8 per cent.

Bis-(6-*diethylaminohexyl*)*ethylamine* (*VIII*, n = 6). 6-Ethylaminohexyldiethylamine (6·5 g.) and 6-chlorohexyldiethylamine[3] (6·3 g.) were refluxed gently in xylene (20 ml.) for 5 hours. On cooling the reaction mixture was extracted with dilute hydrochloric acid (10 per cent) and the latter basified, and extracted with benzene. Evaporation of the solvent yielded an oil (7·7 g.), which on distillation gave after a forerun of starting materials, bis-(6-diethylaminohexyl) ethylamine, as a pale yellow oil, b.p. 165–168°/0·7 mm., n_D^{18} 1·4610 (2·2 g.; 19 per cent). Found: C, 74·7; H, 13·1 per cent. $C_{22}H_{47}N_3$ requires C, 74·3; H, 13·1 per cent.

7:7-*Diethyl*-7-*azoniatridecylenebis* (*triethylammonium*) *triiodide* (IX, $n = 6$). Bis-(6-diethylaminohexyl) ethylamine (0·57 g.) was refluxed with ethyl iodide (3 ml.) for 10 minutes. Evaporation of excess ethyl iodide yielded 7:7-*diethyl*-7-*azoniatridecylenebis* (*triethylammonium*) *triiodide* as colourless needles (from ethanol), m.p. 261–262° (1·02 g.; 77 per cent). Found: N, 4·95; I, 46·2 per cent. $C_{28}H_{64}N_3I_3$ requires N, 5·1; I, 46·2 per cent.

11-*Ethyl*-11-*thioniaheneicosylenebis* (*triethylammonium*) *triiodide* (I, $n = 10$). Bis-10-diethylaminodecyl sulphide[3] (1·1 g.) was refluxed with ethyl iodide (3 to 4 ml.) for 45 minutes. Evaporation of the excess ethyl iodide and recrystallisation of the residual solid from acetone-ether yielded 11-*ethyl*-11-*thioniaheneicosylenebis* (*triethylammonium*) *triiodide* as almost colourless needles, m.p. 123·5–124° (0·6 g.; 27 per cent). Found: N, 3·0; I, 42·0 per cent. $C_{34}H_{75}N_2SI_3$ requires N, 3·0; I, 42·0 per cent.

10-*Bromodecyldiethylamine* (VI, $n = 10$) was prepared from 10-hydroxydecyldiethylamine[3] (20·1 g.) by the method described for 6-bromohexyldiethylamine. 10-*Bromodecyldiethylamine* was obtained as a colourless oil, b.p. 130°/0·5 mm. (21·4 g.; 84 per cent), n_D^{14} 1·4717. Found: equiv. (titration) 294·3; Br, 27·25 per cent. $C_{14}H_{30}NBr$ requires equiv. 292·3; Br, 27·3 per cent.

10-*Ethylaminodecyldiethylamine* (VII, $n = 10$) was prepared from 10-bromodecyldiethylamine (20·9 g.) by the method described for 6-ethylaminohexyldiethylamine. 10-*Ethylaminodecyldiethylamine* was obtained as a colourless oil, b.p. 133–135°/0·8 mm., n_D^{19} 1·4535 (14 g.; 76 per cent). *Dihydrochloride* (from ethanol-ether), m.p. 147–148°. Found: C, 58·1; H, 11·4; Cl, 20·3 per cent. $C_{16}H_{38}N_2Cl_2$ requires C, 58·3; H, 11·6; Cl, 21·5 per cent.

Bis-(10-*diethylaminodecyl*) *ethylamine* (VIII, $n = 10$). 10-Bromodecyldiethylamine (9 g.) in chloroform (10 ml.) was added slowly (40 minutes) to a refluxing solution of 10-ethylaminodecyldiethylamine (7·9 g.) in chloroform (15 ml.), and the mixture refluxed for a further 30 minutes. On evaporation the residue was basified and extracted with benzene. After removal of the solvent, and fractional distillation of the residual oil, *bis*-(10-*diethylaminodecyl*) *ethylamine* was obtained as a pale yellow oil, b.p. 212–216°/0·25 mm., n_D^{14} 1·4660 (3·8 g.; 26·5 per cent). *Trihydrochloride* (from acetone-ether), m.p. 118°. Found: N, 7·1; Cl, 17·8 per cent. $C_{30}H_{68}N_3Cl_3$ requires N, 7·3; Cl, 18·4 per cent.

11 : 11-*Diethyl*-11-*azoniaheneicosylenebis* (*triethylammonium*) *triiodide* (IX, $n = 10$). Bis-(10-diethylaminodecyl) ethylamine (1 g.) was refluxed with ethyl iodide (4 ml.) and ethanol (1 ml.) for 1 hour. Evaporation under reduced pressure gave 11 : 11-*diethyl*-11-*azoniaheneicosylenebis* (*triethylammonium*) *triiodide* (from acetone-ether) as almost colourless crystals, m.p. 202·5–203·5° (1·8 g.; 90 per cent). Found: C, 46·1; H, 8·2; N, 4·4; I, 40·5 per cent. $C_{36}H_{80}N_3I_3$ requires C. 46·2; H, 8·7; N, 4·5; I, 40·7 per cent.

1 : 1-*Bisethoxycarbonyl*-7-*diethylaminoheptane* was prepared from 6-chlorohexyldiethylamine (44 g.) by the method used for the preparation of 1 : 1-bisethoxycarbonyl-4-diethylaminobutane, with the modification that 10 per cent excess sodiomalonic ester was used, and reflux time was increased to 4 hours. 1 : 1-*Bisethoxycarbonyl*-7-*diethylaminoheptane* was obtained as a pale yellow oil, b.p. 147–155°/0·8 mm., $n_D^{15·5}$ 1·4472 (34·5 g.; 47·65 per cent) and used without characterisation in the next stage of the reaction.

Ethyl 8-*diethylaminocaprylate* was prepared from 1 : 1-bisethoxycarbonyl-7-diethylaminoheptane (45·2 g.) by the method used for the preparation of ethyl 5-diethylaminovalerate[1], with the modification that the initial reflux time with hydrochloric acid was increased to 4 hours. *Ethyl* 8-*diethylaminocaprylate*, obtained as a colourless oil, b.p. 111–114°/0·65 mm., n_D^{18} 1·4428 (21·5 g., 62 per cent), was characterised as 7-*ethoxycarbonylheptyl triethylammonium iodide* (prepared by the action of ethyl iodide), m.p. 64·5–65·5° (from acetone-ether). Found: N, 3·6; I, 32·1 per cent $C_{16}H_{34}NO_2I$ requires N, 3·5; I, 32·25 per cent.

8-*Hydroxyoctyldiethylamine*. Ethyl 8-diethylaminocaprylate (35·4 g.) was reduced with lithium aluminium hydride by the method used for the preparation of 5-hydroxypentyldiethylamine[1] to yield 8-*hydroxyoctyldiethylamine* as a colourless oil, b.p. 114–117°/0·7 mm., $n_D^{16·5}$ 1·4590 (26·2 g.; 90 per cent). *Hydrochloride* (from ethanol-ether), m.p. 90–91°.

Found: C, 60·4; H, 11·3; Cl, 15·0 per cent. $C_{12}H_{28}ONCl$ requires C, 60·6; H, 11·9; Cl, 14·9 per cent.

8-*Chlorooctyldiethylamine* was prepared from 8-hydroxyoctyldiethylamine (8·6 g.) by the method described for the preparation of 6-chlorohexyldiethylamine[3]. 8-Chlorooctyldiethylamine was obtained as a colourless oil, b.p. 94–96°/0·55 mm., n_D^{17} 1·4550 (9 g.; 96 per cent). Altman[5] reports b.p. 130·5°/ 11 mm., n_D^{18} 1·4535.

Bis-8-*diethylaminooctyl sulphide* was prepared from 8-chlorooctyldiethylamine (9 g.) by the method described for the preparation of bis-6-diethylaminohexyl sulphide[3]. *Bis*-8-*diethylaminooctyl sulphide* was obtained as a straw-coloured liquid, b.p. 210–212°/0·65 mm., $n_D^{16·5}$ 1·4768 (5·9 g.; 72 per cent). Found: equiv. (titration) 203·5. $C_{24}H_{52}N_2S$ requires equiv. 200·4. *Dihydrochloride* (from ethanol), m.p. 145°. Found: C, 60·85; H, 11·0 per cent. $C_{24}H_{54}N_2SCl_2$ requires C, 60·85; H, 11·5 per cent.

9-*Ethyl*-9-*thioniaheptadecylenebis* (*triethylammonium*) *triiodide*. Bis-8-diethylaminooctyl sulphide (0·63 g.) was refluxed with ethyl iodide (4 ml.) for 15 minutes. Removal of excess reagent under reduced pressure yielded 9-*ethyl*-9-*thioniaheptadecylenebis* (*triethylammonium*) *triiodide* (0·64 g.; 47 per cent) (from ethanol-ether), m.p. 159–160° (decomp.). Found: N, 3·2; S, 3·85, I, 43·0 per cent. $C_{30}H_{67}N_2SI_3$ requires N, 3·2; S, 3·7; I, 43·8 per cent.

8-*Ethylaminooctyldiethylamine* was prepared from 8-hydroxyoctyldiethylamine (8·6 g.) by the method described for the preparation of 6-ethylaminohexyldiethylamine. 8-*Ethylaminooctyldiethylamine* was obtained as a colourless oil, b.p. 104–106°/0·7 mm., $n_D^{17·5}$ 1·4530 (7·4 g.; 76 per cent). *Dihydrochloride* (from ethanol-ether), m.p. 159·5–160·5° (hygroscopic). Found: N, 9·2; Cl, 23·0 per cent. $C_{14}H_{34}N_2Cl_2$ requires N, 9·3; Cl, 23·5 per cent.

Bis-(8-*diethylaminooctyl*) *ethylamine* was prepared from 8-ethylaminooctyldiethylamine (6·95 g.) and 8-chlorooctyldiethylamine (6·7 g.) by the method described for the preparation of bis-(6-diethylaminohexyl) ethylamine. *Bis*-(8-*diethylaminooctyl*) *ethylamine* was obtained as a yellow oil, b.p. 230–250° (bath)/0·8 mm., n_D^{17} 1·4642 (2·3 g.; 18 per cent). *Trihydrochloride* (from acetone-ether), m.p. 165–166° (decomp.). Found: Cl, 20·9 per cent. $C_{26}H_{60}N_3Cl_3$ requires Cl, 20·4 per cent.

9 : 9-*Diethyl*-9-*azoniaheptadecylenebis* (*triethylammonium*) *triiodide*. Bis-(8-diethylaminooctyl) ethylamine (0·56 g.) was refluxed with ethyl iodide (2 ml.) and ethanol (2 ml.) for 15 minutes. On evaporation under reduced pressure 9 : 9-*diethyl*-9-*azoniaheptadecylenebis* (*triethylammonium*) *triiodide* was obtained (from ethanol), m.p. 251–252° (decomp.). Found: C, 43·2; H, 8·1; I, 43·0 per cent. $C_{32}H_{72}N_3I_3$ requires C, 43·7; H, 8·25; I, 43·3 per cent.

7-*Dioxothiatridecylenebis* (*triethylammonium iodide*). Bis-6-diethylaminohexyl sulphone[3] (0·99 g.) was refluxed with ethyl iodide (3 ml.) for 2 hours, when a brownish oil separated from the reaction mixture. After evaporation of excess ethyl iodide, the oil was treated in water with charcoal, and the solution evaporated to dryness. The oily residue

crystallised from acetone-ether gave *7-dioxothiatridecylenebis (triethylammonium iodide)* as a pale buff solid, m.p. 144–145° (0·85 g.; 47 per cent). Found: C, 41·5; H, 7·9; N, 3·9 per cent. $C_{24}H_{54}O_2N_2SI_2$ requires C, 41·9; H, 7·9; N, 4·1 per cent.

PHARMACOLOGICAL

Materials. The following were used: tris-onium derivatives, dihexazonium triethiodide (DHAE), dioctazonium triethiodide (DOAE) and didecazonium triethiodide (DDAE); tris-sulphonium derivatives, dioctasulphonium triethiodide (DOSE) and didecasulphonium triethiodide (DDSE); sulphone (bis-onium) dihexone. Drugs used were: decamethonium iodide (C 10), tubocurarine chloride (TC), edrophonium chloride (edrophonium), eserine salicylate (eserine), neostigmine methylsulphate (neostigmine), adrenaline hydrochloride, (Ad), potassium chloride (KCl), ether, acetylcholine chloride (ACh), sodium pentobarbitone and atropine sulphate (atropine).

Methods and results. The experimental methods used in this investigation were similar to those described by us in an earlier publication[1].

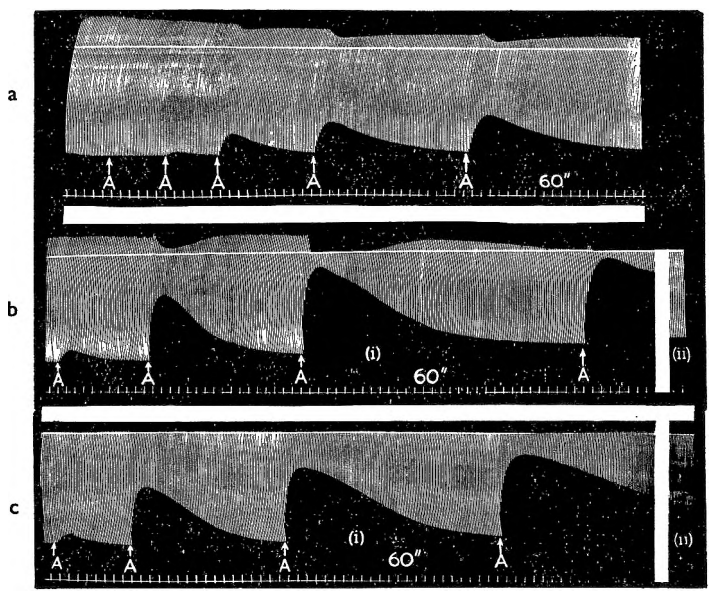


FIG. 1. Cat gastrocnemius-sciatic preparation. Pentobarbitone anaesthesia. Indirect stimulation via sciatic nerve. Contraction downwards. Drugs administered intravenously.

(a) At A, tubocurarine 0·075 mg./kg.
(b) At A, dioctazonium 0·30 mg./kg.
There was an interval of 40 minutes between (i) and (ii).
(c) At A, dihexazonium 0·20 mg./kg.
There was an interval of 25 minutes between (i) and (ii).

Neuromuscular blocking activity. Cats of either sex, weighing between 2 and 4 kg. were anaesthetised by intraperitoneal injection of sodium pentobarbitone (about 60 mg./kg.). The preparation was set up for recording the contractions of the gastrocnemius muscle in response to indirect stimulation *via* the sciatic nerve. A Dobbie-McInnes stimulator was used to deliver supra-maximal square impulses at a frequency of 4 to 8 per minute, pulse width 1·5 to 3·0 msec., voltage 12 to 20 volts. In any one experiment, frequency, pulse width and voltage were constant except when indirectly tetanising the muscle when the frequency was 1,500 per minute, or when stimulating the muscle directly when the voltage was raised to 40 volts.

Dihexazonium triethiodide, dioctazonium triethiodide, dioctasulphonium triethiodide and dihexone in the dose range 0·10 to 1·0 mg./kg. caused a reduction of the amplitude of the twitch but no initial potentiation of twitch height, muscular twitching or fibrillation were seen. In the case of dihexazonium triethiodide a dose of 0·2 to 0·4 mg./kg. was adequate to give an approximately 95 per cent reduction in twitch height. Smaller doses produced graded but reduced effects. The effect of the initial dose was usually small but that of the second, third and often of the fourth dose was progressively greater. The effect upon twitch height then became approximately constant although the duration of effect increased with successive doses. Similar effects were seen when TC was used at similar

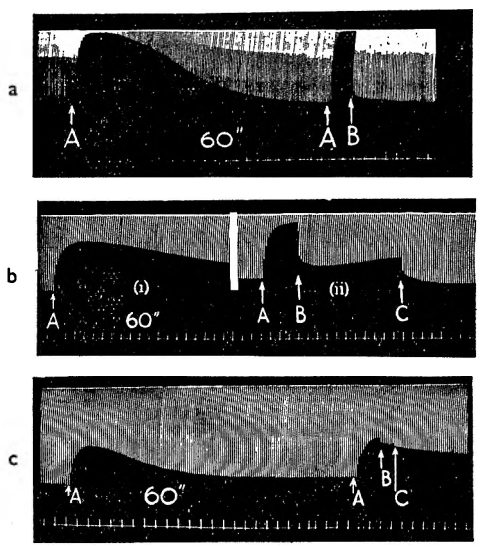


FIG. 2. Cat gastrocnemius-sciatic preparation. Pentobarbitone anaesthesia. Indirect stimulation via sciatic nerve. Contraction downwards. Drugs administered intravenously.

(a) At A, dihexazonium 0·40 mg./kg.
At B, edrophonium 0·40 "
(b) At A, dioctazonium 0·20 "
At B, edrophonium 0·50 "
At C, edrophonium 1·0 "
(c) At A, dihexone 1·50 mg./kg.
At B, edrophonium 0·50 "
At C, edrophonium 0·75 "

dose levels (Fig. 1). Dihexazonium triethiodide and TC appeared to be about equipotent whilst dioctazonium triethiodide, dioctasulphonium triethiodide and dihexone had properties qualitatively idential with those of dihexazonium but were less potent. Dioctazonium triethiodide had about two-thirds of the potency of TC, dioctasulphonium triethiodide one-half and dihexone one-eighth. The neuromuscular blocking activity of dihexazonium triethiodide, dioctazonium triethiodide, dioctasulphonium triethiodide and dihexone was antagonised by edrophonium (0·5 to 1·0 mg./kg.). In the case of dioctazonium triethiodide and dihexazonium triethiodide recovery of the twitch height was prompt and complete, but after dioctasulphonium triethiodide and dihexone edrophonium did not produce complete recovery (Fig. 2). Neostigmine (0·05 to 0·10 mg./kg.) had similar effects, but eserine (0·5 to 1·0 mg./kg.) was much less effective. The effects of these four blocking agents were partially antagonised by Ad (0·05 to 0·1 mg./kg.), KCl (15 to 20 mg./kg.) and C 10 (0·10 to 0·40 mg./kg.). Some of these effects are shown in

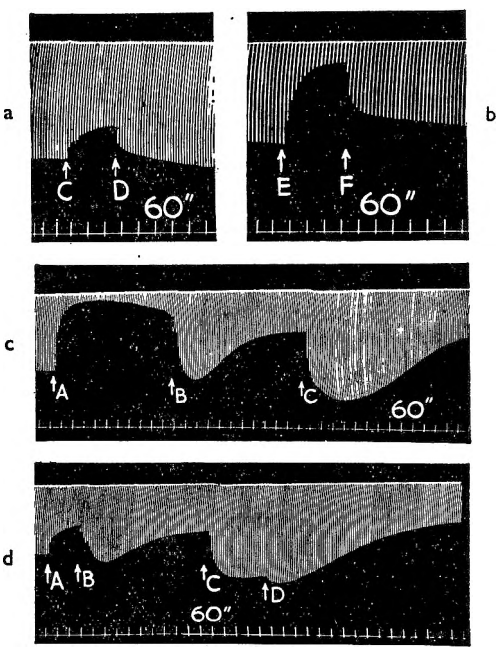


FIG. 3. Cat gastrocnemius-sciatic preparation. Pentobarbitone anaesthesia. Indirect stimulation via sciatic nerve. Contraction downwards. Drugs administered intravenously.

(a)	At C, dioctasulphonium	0·15 mg./kg.	(d)	At A, dihexazonium	0·40 mg./kg.
	At D, decamethonium	0·07 "		At B, potassium chloride	20 "
(b)	At E, dihexone	0·40 "		At C, adrenaline	0·10 "
	At F, decamethonium	0·06 "		At D, adrenaline	0·05 "
(c)	At A, dihexone	0·40 "			
	At B, potassium chloride	20 "			
	At C, adrenaline	0·08 "			

Figure 3. C 10 (0·10 to 0·40 mg./kg.) rapidly reversed the neuromuscular block caused by dihexazonium triethiodide (0·10 to 0·30 mg./kg.), dioctazonium triethiodide (0·20 to 0·80 mg./kg.), dioctasulphonium triethiodide (0·20 to 0·80 mg./kg.) or dihexone (1·0 to 2·0 mg./kg.). The effects of all four compounds were potentiated by ether and it was found that if the partially blocked muscle was indirectly tetanised the tetanic response was poorly sustained following dihexone, dioctazonium triethiodide and dihexazonium triethiodide but after dioctasulphonium triethiodide it was well maintained (Fig. 4). When the muscle had become unresponsive to indirect stimulation, direct stimulation caused it to contract, but the response did not attain the same amplitude as the control. It was usually possible to obtain a response of 60 to 80 per cent of the control level, but not more. Partial, but temporary decurarisation was seen to follow indirect tetanisation of the muscle in preparations partly blocked by dihexazonium triethiodide, dioctazonium triethiodide, dioctasulphonium triethiodide or dihexone. The influence of tetanisation upon the effects of these drugs is compared in Figure 4 with its effect upon TC blockade which is influenced in a similar fashion.

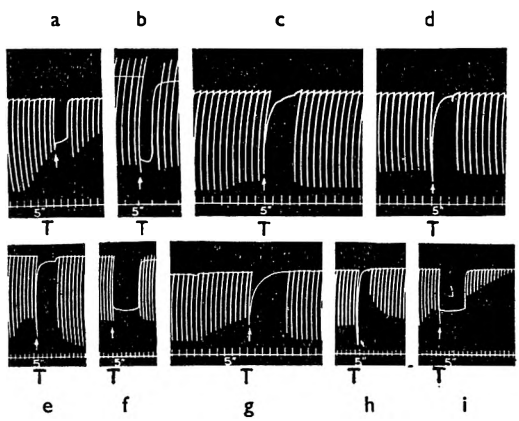


FIG. 4. Cat gastrocnemius-sciatic preparation. Pentobarbitone anaesthesia. Contraction downwards. Drugs administered intravenously. At T, indirect tetanization via sciatic nerve during partial block by (a) dioctasulphonium, (b) didecazonium, (c) dioctazonium, (d) didecasulphonium, (e) dihexazonium, (g) tubocurarine, (h) dihexone, (i) decamethonium and tetanization of the normal muscle (f).

Didecasulphonium triethiodide and didecazonium triethiodide had properties different from those of the drugs already described. Didecazonium triethiodide in the dose range of 0·01 mg./kg. to 0·20 mg./kg. caused initial potentiation of twitch height (Fig. 5) but didecasulphonium triethiodide did not share this property. Potentiation of the twitch height with didecazonium triethiodide was followed by a progressive decline and this was accompanied in its early stages by a generalised, intermittent muscular twitching and fasciculation. Didecasulphonium triethiodide (0·20 to 0·50 mg./kg.) also caused neuromuscular block but this was accompanied by generalised muscular twitching. Both didecasulphonium triethiodide and didecazonium triethiodide caused a very

prolonged neuromuscular block (Fig. 5); during complete neuromuscular paralysis with didecasulphonium triethiodide the response to direct stimulation was very poor, but with didecazonium triethiodide it was as much as 40 per cent of the control height. After didecazonium triethiodide (0·20 mg./kg.) the response of the partially blockaded muscle to indirect tetanization was well maintained but following didecasulphonium triethiodide (0·25 mg./kg.) it waned rapidly and decurarisation did not follow indirect tetanisation (Fig. 4). Neuromuscular blockade with didecasulphonium triethiodide (0·2 to 0·4 mg./kg.) was slightly reversed by neostigmine (0·10 to 0·20 mg./kg.) and edrophonium (1·0 to 2·0 mg./kg.), but eserine (0·5 to 2·0 mg./kg.) had no antagonistic effect. Ad (0·05 to 0·1 mg./kg.) antagonised the effects of didecasulphonium as did KCl (20 mg./kg.).

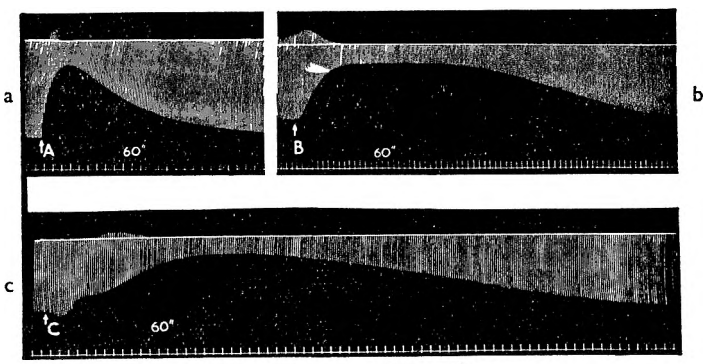


FIG. 5. Cat gastrocnemius-sciatic preparation. Pentobarbitone anaesthesia. Indirect stimulation via sciatic nerve. Contraction downwards. Drugs administered intravenously.

(a) At A, tubocurarine 0·25 mg./kg.
(b) At B, didecasulphonium 0·30 "
(c) At C, didecazonium 0·25 "

Antagonism to the actions of didecazonium triethiodide was not shown by eserine (0·5 to 2·0 mg./kg.) or by neostigmine (0·10 to 0·20 mg./kg.) and its effects were potentiated by edrophonium (1·0 to 2·0 mg./kg.) and antagonised by Ad (0·05 mg./kg.) and KCl (20 mg./kg.). Ad had a temporary, but well marked antagonism to the neuromuscular blocking effects of all of the six compounds which we have investigated as well as to the effects of TC and C 10. The effects of didecasulphonium triethiodide or didecazonium triethiodide were not potentiated by ether anaesthesia. The neuromuscular blocking effects of didecasulphonium triethiodide and didecazonium triethiodide were additive with those of C 10 and these compounds antagonised the neuromuscular blocking effects of TC.

Rabbit head drop. Comparisons of potency were made with TC using a slightly modified rabbit head drop method and the experiments were repeated in rabbits which had been given neostigmine (0·1 mg./kg.) by

subcutaneous injection 15 minutes before. The results are shown in columns 1 and 2 of Table I. Column 3 shows the ratio of the two head drop doses. This ratio is some indication of the mode of drug action; if it is greater than unity it indicates drug antagonism and hence a TC-like effect; if less than unity it suggests synergism or no antagonism which indicates a C 10-like mode of action.

TABLE I

RABBIT HEAD DROP DOSES (H.D.D.)

Compound	H.D.D. ± s.e. (mg./kg.)	H.D.D. after neostigmine ± s.e. (mg./kg.)	Ratio H.D.D. after neostigmine/ H.D.D.
Dihexazonium	0·51 ± 0·057	0·81 ± 0·057	1·56 (P = <0·01)
Dioctasulphonium	0·24 ± 0·013	0·25 ± 0·013	1·04 (P = >0·90)
Dioctazonium	0·26 ± 0·014	0·33 ± 0·014	1·27 (P = <0·01)
Didecasulphonium	0·21 ± 0·023	0·28 ± 0·023	1·30 (P = >0·05)
Didecazonium	0·28 ± 0·017	0·28 ± 0·017	1·00
Dihexone	0·39 ± 0·017	0·44 ± 0·017	1·12 (P = >0·40)
Decamethonium	0·16 ± 0·008	0·15 ± 0·008	0·93
Tubocurarine	0·11 ± 0·01	0·30 ± 0·01	2·72 (P = <0·01)

Acute toxicity in mice. Using groups of ten mice the approximate LD50 for the six compounds listed was found and compared with that found for TC and C 10. The results are summarised in Table II.

TABLE II

ACUTE TOXICITY (LD50) AND PARALYSING ACTIVITY (PD50) IN MICE

Compound	Approximate LD50 Groups of 10 mice (mg./kg.)	Approximate PD50 Groups of 10 mice (mg./kg.)	Paralysing potency TC = 100
Dihexazonium	2·25	1·20	17
Dioctasulphonium	0·40	0·08	250
Dioctazonium	0·40	0·10	200
Didecasulphonium	0·20	0·10	200
Didecazonium	0·18	0·16	125
Dihexone	2·30	0·60	33
Decamethonium	4·10	3·65	5·4
Tubocurarine	0·28	0·20	100

Paralysing activity in mice. Mice were given an intraperitoneal injection of the drug and placed upon the upper part of a fine wire mesh screen which was inclined at an angle of 60° to the horizontal. This method was used by Thompson[6] for an insulin assay in mice. Groups of ten mice were used at each dose level and four dose levels were used for each compound. The number of mice in each group which were unable to maintain their position on the screen at each dose level was counted and from this an approximate 50 per cent paralysing dose (PD50) was calculated. The results obtained are summarised in Table II.

Effects upon blood pressure and respiration. The effects of didecazonium triethiodide, dioctazonium triethiodide, dihexazonium triethiodide, dioctasulphonium triethiodide, didecasulphonium triethiodide and dihexone on the blood pressure of cats anaesthetised with sodium pentobarbitone were compared with the effects of TC (0·5 to 1·0 mg./kg.). Dose levels of up

to 2 mg./kg. had no significant effects upon the level of the blood pressure but 1 mg./kg. of TC caused a typical prolonged fall. The average doses required to paralyse respiration in the pentobarbitone-anaesthetised cats are shown in Table III. In these experiments, a solution of the drug which contained 0·2 mg./ml. was infused into the external jugular vein using a Palmer's constant rate infusion apparatus at a rate of 0·8 ml./minute.

TABLE III

RESPIRATORY PARALYSING ACTIVITY IN THE ANAESTHETISED CAT

Compound	Mean respiratory paralysing dose (mg./kg.)	Potency (TC = 100)
Dihexazonium	0·85	56
Dioctasulphonium	0·93	51
Dioctazonium	1·24	38
Didecasulphonium	1·19	40
Didecazonium	0·57	84
Dihexone	1·70	28
Decamethonium	0·15	320
Tubocurarine	0·48	100

Ganglion blocking activity. Ganglion blocking activity was investigated by noting the effects of the intravenous injection of 0·5 to 2·0 mg./kg. of the drug upon the response of the nictitating membrane of the pentobarbitone anaesthetised cat to stimulation of the pre-ganglionic fibres of the cervical sympathetic. A Dobbie-McInnes stimulator was used to deliver 15-second bursts of square impulses at a frequency of 800 to 1,200 per minute and at 10 volts. In any one experiment frequency was kept constant. TC (0·5 mg./kg.) caused a typical well marked depression of the amplitude of response but dihexazonium triethiodide, didecasulphonium triethiodide, dioctazonium triethiodide, dioctasulphonium triethiodide and dihexone (up to 2·0 mg./kg.) did not reduce the height of the contraction unless given at very high dose levels. Didecazonium triethiodide appeared to be a ganglion stimulant since after an injection

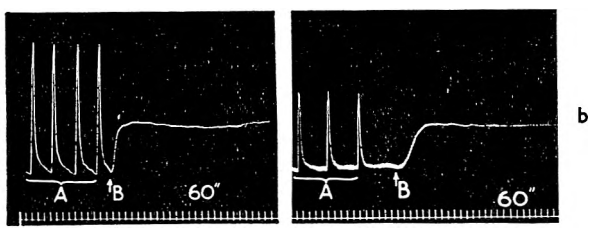


FIG. 6. Cat. Pentobarbitone anaesthesia.

(a) At A, contractions of nictitating membrane elicited by pre-ganglionic stimulation of the cervical sympathetic at 12 volts, 1,000 impulses per minute, pulse width 1·0 msec. bursts of 15 seconds.
At B, didecazonium 0·50 mg./kg. intravenously.

(b) At A, contractions of nictitating membrane elicited by pre-ganglionic stimulation of the cervical sympathetic at 15 volts, 800 impulses per minute, pulse width 1·5 msec. bursts of 15 seconds.
At B, infusion of didecazonium (0·20 mg./ml.) at the rate of 0·80 ml./minute for a period of 10 minutes.

of 0·4 mg./kg. of this drug there was a well marked contraction of the nictitating membrane (Fig. 6).

Frog rectus abdominis muscle. The muscle was set up in a 20 ml. bath containing oxygenated frog Ringer's solution at room temperature[1]. No direct stimulant actions were seen when any of the six compounds were added to the bath at dose levels of up to 25 μg./ml. All of the compounds (1·0 to 5 μg./ml.) antagonised contractures due to ACh (0·10 to 0·25 μg./ml.) and C 10 (1·5 to 2·5 μg./ml.), but in one experiment only, didecazonium triethiodide (2·0 to 5 μg./ml.) caused a slow contracture-like response of the rectus.

Isolated guinea pig ileum. The ileum was set up in a 2 ml. bath of oxygenated Tyrode's solution at 29°[1]. Slight antagonism was shown to contractions induced by ACh (0·10 to 0·5 μg./ml.) and ACh-induced contractions were not potentiated. All the compounds showed direct stimulant properties when given at large dose levels (0·1 to 0·2 mg./ml.) and the contractions of the ileum were blocked by atropine (0·05 μg./ml.).

Isolated rabbit duodenum. The duodenum was set up in a 50 ml. bath of oxygenated Locke's solution at 37°[1]. All of the compounds caused a contraction of the duodenum (10 to 40 μg./ml.). C 10 caused similar contractions whilst TC had no direct effect. The stimulant effects were blocked by atropine (0·004 μg./ml.).

Isolated rat hindquarters. The rat hindquarters were set up and perfused with oxygenated Locke's solution at room temperature according to the method described by Burn[7]. Variations in outflow were recorded by means of a Gaddum drop recorder. None of the compounds in the dose range of 0·25 to 1·0 mg. caused any significant alteration in the outflow of the perfusion fluid, but in this dose range tubocurarine usually caused constriction of the blood vessels.

Rat and kitten phrenic nerve-diaphragm preparations. Comparisons of potency were made on both the rat and kitten diaphragm. The rat diaphragm was set up according to the method of Bülbring[8] and a similar technique was used to set up the kitten diaphragm. A 100 ml. bath of oxygenated "double glucose" Tyrode's solution at 29° was used. The nerve was stimulated by means of a Dobbie-McInnes stimulator, using a Collison's fluid electrode. The frequency of stimulation was 8 per minute, pulse width 3 msec. at 15 volts. The approximate potency ratios (TC = 100) in the rat diaphragm were as follows:

Dihexazonium triethiodide 2; dihexone 0·67; didecasulphonium triethiodide 2; dioctazonium triethiodide 1·4; dioctasulphonium triethiodide 3; dioctazonium triethiodide 2. Using the kitten diaphragm these became (TC = 100): dihexazonium triethiodide 400; dihexone 6; didecasulphonium triethiodide 14; didecazonium triethiodide 14; dioctasulphonium triethiodide 200; dioctazonium triethiodide 150.

We can offer no explanation for this marked species difference.

Discussion

All the compounds tested possess neuromuscular blocking activity. The *NSN*-tris-onium compound dioctasulphonium triethiodide (DOSE;

I, $n = 8$, R = Et), the *NNN*-tris-onium compounds dihexazonium triethiodide (DHAE; IX, $n = 6$) and dioctazonium triethiodide (DOAE; IX, $n = 8$), and the bis-quaternary sulphone (IV) have TC-like properties in almost every respect, although when dioctasulphonium is used to induce partial neuromuscular blockade in the cat gastrocnemius muscle-sciatic nerve preparation and the muscle is then indirectly tetanised, the tetanus is well maintained. This is a property shared with C 10 and with the *NNN*-tris-quaternary didecazonium triethiodide (DDAE; IX, $n = 10$), but not with the *NSN*-tris-onium compound didecasulphonium triethiodide (DDSE; I, $n = 10$, R = Et). Both didecasulphonium and didecazonium show predominantly C 10-like activity.

If the properties of dipentasulphonium and dihexasulphonium are taken into account it is clear that as the interquaternary chain-length increases, members of the *NSN*-series change from being TC-like (non-depolarising) in those compounds with five and six-membered polymethylene chains, becoming first transitional in compounds with eight methylene units, and then predominantly C 10-like (depolarising) when the methylene chain is extended to ten units. Within the series of compounds tested the nature of the third (central) onium group in the tris-onium neuromuscular blocking agents appears to be relatively unimportant in determining the type of activity produced. Replacement of sulphur by nitrogen gives a series of *NNN*-tris-quaternary compounds which show the same broad gradation of properties observed with the analogous *NSN*-compounds, although small differences are evident. Thus the neuromuscular blocking activity of didecasulphonium is not always increased by edrophonium and the tension of an indirect tetanus in the partly blockaded muscle is not well sustained. Didecazonium, on the other hand, possesses an almost classic type of depolarising activity although it does not usually stimulate the frog rectus muscle.

If we exclude from discussion all question of the influence of *N*- or *S*-alkyl group size, it is evident from the results of our experiments that chain extension with the interpolation of a third onium group leads to a pronounced enhancement of the TC-like properties compared with that observed in the bis-quaternary series. Thus, in contrast to dihexasulphonium and dihexazonium, which are approximately equipotent with TC, hexamethonium only causes a TC-like neuromuscular blockade when given at very high dose levels. No such distinction, however, can be drawn between the bis- and tris-quaternary series in regard to depolarising properties which in both only reach significant proportions when the number of methylene groups separating the charged centres is of the order of ten units.

The importance of the third (central) onium group, and the number of units in the polymethylene chain separating the individual quaternary centres, rather than overall molecular chain length, as factors in determining the level of TC-like activity, follows from the much higher potency of dihexasulphonium and dihexazonium as compared with that of the bis-quaternary sulphone (IV) and bis-quaternary compound decaethonium. It is important, however, to distinguish between the number of units in

the polymethylene chain as a measure of interonium group distance within a molecule and the actual distance which separates these centres at the moment of combination with receptors at the site of action. The essential linearity of the individual polymethylene chains of the *NNN*-compounds, both in the solid state and in solution will be favoured by the natural repulsion of the individual positively charged heads within each molecule, and by the tetrahedral nature of quaternary nitrogen. Substitution of one quaternary nitrogen by tertiary sulphur (which is pyramidal) in the *NSN*-compounds should not appreciably affect the shape of the molecules since the C–$\overset{+}{S}$–C valency angle differs but little from that of C–$\overset{+}{N}$–C group. In consequence no appreciable difference between the properties of comparable members of the two series is expected or found. Although we are unable at present to ascertain whether or not these molecules retain their linearity at the site of action there is some evidence from studies of the bis-quaternary compounds that this may be so. Thus, for example, TC, in which the molecule is more or less completely rigid, and decaethonium have the same interquaternary distance only if the latter molecule is assumed to be linear, and both show the same *type* of activity (note that our results link *type* of activity strongly with inter-onium group spacing) albeit of significantly differing potency.

The size of the *N*- or *S*-alkyl groups as a factor in determining the level and type of neuromuscular blocking activity is not clear. In gallamine, a tris-ethonium derivative, replacement of *N*-ethyl by *N*-methyl groups reduces the activity but does not alter its character[9]. With decamethonium on the other hand, replacement of methyl by ethyl groups not only reduces activity but changes its character[10–11]. The effect of *N*- or *S*-alkyl group size has not yet been fully investigated in the present series. All the compounds tested have been ethonium salts, although we have previously reported[1] that dihexasulphonium trimethiodide is almost devoid of neuromuscular blocking activity, an observation which suggests that these compounds fall into the same category as gallamine.

The reason for this reduction in activity is not clear, but we hope to investigate the properties of the methonium analogues of the entire series at a later date.

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NEUROMUSCULAR BLOCKING AGENTS

PART III. SOME LINEAR *NNNN*-TETRA-ETHONIUM COMPOUNDS

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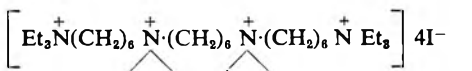
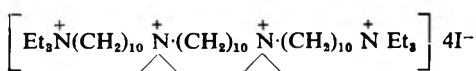
IN extension of our observation of neuromuscular blocking activity in tris-onium compounds reported in Part I[1] and Part II[2] we have now prepared the linear tetra-azonium compounds, 7:7:14:14-tetraethyl-7:14-diazoniaeicosylenebis(triethylammonium) tetraiodide (Trihexatetrazonium tetraethiodide; THAE; I), m.p. 248–248·5° (Found: N, 4·9; I, 45·9 per cent. $C_{38}H_{86}N_4I_4$ requires N, 5·1; I, 45·9 per cent) and 11 : 11 : 22 : 22 - tetraethyl - 11 : 22 - diazoniadotriacontylenebis(triethylammonium) tetraiodide (Tridecatetrazonium tetraethiodide; TDAE; II), m.p. 186–187° (Found: C, 46·8; H, 8·5; I, 39·4 per cent. $C_{50}H_{110}I_4N_4$ requires C, 47·1; H, 8·7; I, 39·8 per cent).



(I)



(II)

The experimental methods and materials have been described in detail in our previous publications[1,2]. The neuromuscular blocking actions of I were similar to those of tubocurarine (TC). For example, I (0·03 to 0·06 mg./kg.) caused a reduction of twitch amplitude of the gastrocnemius muscle of the cat excited indirectly *via* the sciatic nerve. There was no initial potentiation of twitch height and no muscular twitching or fasiculation. On the cat, I was about three times as potent as TC and its neuromuscular blocking activity was antagonised by edrophonium (0·5 to 1·0 mg./kg.), neostigmine (0·6 to 0·1 mg./kg.), eserine (0·5 to 1·0 mg./kg.), adrenaline (0·05 to 0·1 mg./kg.), potassium chloride (20 mg./kg.) and decamethonium (C 10) (0·02 to 0·6 mg./kg.). The effects of I were additive with those of TC and were potentiated by ether. The muscle partially blockaded by I (0·02 mg./kg.) was unable to maintain an indirect tetanus but when the muscle had become unresponsive to indirect stimulation, direct stimulation caused a contraction.

The properties of II were quite different from those of I and TC. It was noticed that 0·2 to 0·4 mg./kg. of II caused marked muscular twitching; the movements were at times almost convulsive with apparent

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involvement of all of the skeletal muscles. Twitch amplitude was reduced but maximum depression of twitch height was not obtained until 10 to 20 minutes after the dose had been given. The effects were prolonged and the response after one dose of 0·2 mg./kg. did not return to control levels for about 1 hour. A second and similar dose caused a more prolonged depression and recovery took from 2 to 3 hours. The neuromuscular blocking actions of II were not antagonised by eserine (1 mg./kg.) or neostigmine (0·1 mg./kg.) whilst 0·6 to 1·0 mg./kg. of edrophonium potentiated it. The effects of II were additive with those of C 10, whilst TC antagonised its actions. When the muscle had become unresponsive to indirect stimulation direct stimulation caused only a very small response. The response to indirect tetanisation of the muscle was fairly well maintained but not comparable with the response of the untreated or C 10-treated preparation (Fig. 1).

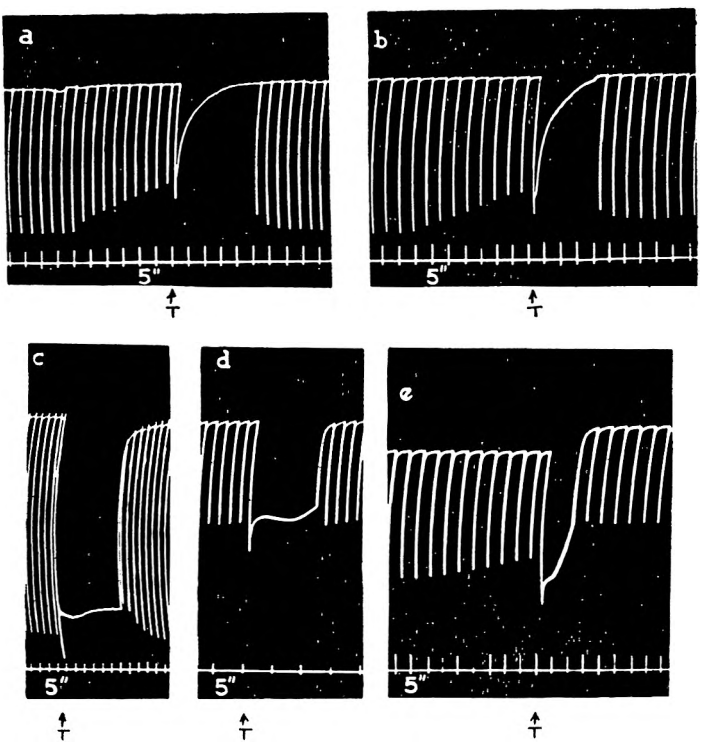

FIG. 1. Cat. Gastrocnemius-sciatic preparation. Pentobarbitone anaesthesia. Contraction downwards. Drugs administered intravenously. At T, indirect tetanisation of the gastrocnemius *via* the sciatic nerve during partial block by (*a*) tubocurarine, (*b*) trishexatetrazonium, (*d*) decamethonium, (*e*) trisdecatetrazonium, and (*c*) normal muscle.

The head drop doses of I and II both before and after treatment with neostigmine have been obtained and compared with TC. The figures are shown in Table I and indicate that I probably acts in a similar manner to

TC, whilst II has a different mechanism of action and appears to resemble C 10.

TABLE I

A COMPARISON OF THE HEAD DROP DOSES OF TRIHEXATETRAZONIUM (I) AND TRIDECATETRAZONIUM (II) WITH TUBOCURARINE IN THE RABBIT BEFORE AND AFTER TREATMENT WITH NEOSTIGMINE

Compound	Mean head drop dose ± s.e. (mg./kg.)		Potency	Ratio: neostigmine treated/control
	Control	After neostigmine (0·10 mg./kg.)		
Trihexatetrazonium tetra-ethiodide ..	0·19 ± 0·014	0·31 ± 0·014	58	1·63
Tridecatetrazonium tetra-ethiodide ..	0·40 ± 0·024	0·33 ± 0·024	28	(P = 0·01) 0·82
Tubocurarine	0·11 ± 0·01	0·30 ± 0·01	100	2·72 (P = 0·01)

I (1·0 to 2·0 mg./kg.) was found to have no significant effect upon the arterial blood pressure level of anaesthetised cats, but II (1·0 to 2·0 mg./kg.) caused a moderate, fairly prolonged fall and its effects were similar to those usually seen when TC (1·0 mg./kg.) is used (Fig. 2). In contrast to TC (0·5 mg./kg.), I and II in doses of up to 2·0 mg./kg. had no effect upon the response of the nictitating membrane of the anaesthetised cat to stimulation of the preganglionic fibres of the cervical sympathetic.

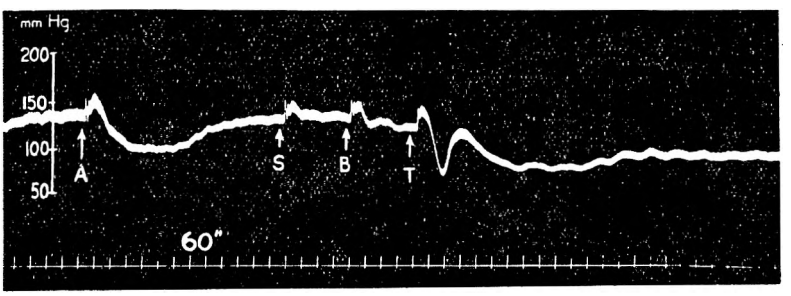

FIG. 2. Cat. Pentobarbitone anaesthesia. Blood pressure record from the common carotid artery. Drugs administered intravenously and in each instance followed by 4 ml. saline. At A, trisdecatetrazonium 1·0 mg./kg. At S, saline 4 ml. At B, trishexatetrazonium 1·0 mg./kg. At T, tubocurarine 1·0 mg./kg.

Our experiments show that the properties of the tetraethonium compounds I and II are similar to those of the corresponding tris-ethonium compounds which we have investigated[1,2], in that inter-onium group chain length is a major factor in determining the type of neuromuscular blocking activity. Thus, when the quaternary centres are separated by polymethylene chains containing five or six units[1,2], activity is virtually purely TC-like. Increase of the inter-quaternary chain length to eight methylene units[2] leads to activity which is predominantly TC-like, but some C 10-like activity appears, whilst compounds with polymethylene chains of ten

units show activity which closely resembles that of C 10. It is of interest that these linear-*NNNN*-compounds are more potent than the corresponding *NNN*- and *NSN*-derivatives, and we are investigating the effect of increasing the number of quaternary centres still further.

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DISCUSSION

The paper and short communication were presented by MR. J. J. LEWIS.

THE CHAIRMAN. Had the compounds anticholinesterase activity?

DR. G. F. SOMERS (Liverpool). The use of a mixed muscle, the gastrocnemius, was open to criticism; the tibialis and soleus of the cat, or avian muscle, would have been useful in distinguishing depolarising action from competitive block.

DR. F. HARTLEY (London). Had differences been found according to the concentration use? If potency increased as the chain lengthened, the effect was to double the concentration with the di-onium derivatives. What was the effect of asymmetry in the tris compounds?

DR. A. H. BECKETT (London). The stereochemistry of the compounds should be considered. With a branched chain in the vicinity of the nitrogen the isomers could be examined critically. There was little information about the spatial arrangements of isomers with different biological action. Was work being done on this? If a desired biological effect was present, instead of large molecular changes, there should be a three-dimensional approach, with small molecular changes.

MR. LEWIS replied. In neuromuscular blocking agents there was great species variation in mode and type of action. Other cat muscles had been used, and results on avian muscle were in line with those reported, but the cat was a quantitative rather than qualitative preparation. The type of action was independent of the concentration. With a chain length of eight the action was still predominantly tubocurare-like; only in toxicity was there a close relationship to decamethonium. Although *NNN* with six carbons between the nitrogen was like two hexamethonium molecules it did not behave as such. This indicated a fundamental difference in the type of action between ganglionic and neuromuscular blocking agents. They intended to interest themselves in the more fundamental inter-onium chain lengths before investigating the stereochemical problem. The action of rigid molecules with limited steric changes or rotation was of considerable interest. The *NSN* and *NNN* compounds, dihexasulphonium and dihexazonium, which were equi-potent and had the same activity in the cat and other animals, had considerable differences in man. The change of S to N made a great difference in potency in man. Anticholinesterase activity had not been directly tested. Six different species

of animals had been used, and there was variation. The tris $N(CH_2)_6$ compound was equipotent with gallamine in man but with tubocurare in cat. The analogue with a middle sulphur although equipotent with tubocurare in the cat had only $\frac{1}{6}$ to $\frac{1}{8}$ the potency of gallamine in man.

DR. STENLAKE replied. No work had been done on asymmetric compounds in the tris series, but in the tetra-onium series, compounds had been prepared where the inter-onium units were 6 : 10 : 6 and 10 : 6 : 10. The former was curare-like and the latter predominantly depolarising.

SOME ASPECTS OF THE PROPERTY OF ANGLE OF REPOSE OF POWDERS

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Four methods of determining the angle of repose of free flowing powders have been critically compared using graded samples of glass balls, lead shot and silver sand. The method used influenced the result. Results of all methods have been correlated graphically and values for static and dynamic interparticulate friction have been assessed for the materials used. It was found that results for spheres could be correlated on one graph, irrespective of type of material and an explanation is given to account for this.

An intrinsic property of any powder is its resistance to differential movement between particles when subjected to external forces. Probably this property should be best described as *interparticulate friction*, of which the *angle of repose* is a manifestation. Practical methods[1–7] of assessment of interparticulate friction are mainly based on the measurement of the angle of repose of the loose powder mass. There would appear to be four main methods of measuring the angle of repose and, for comparison, the essentials of these methods are set out in the line diagrams in Figure 1.

Method I. Fixed Funnel and Free Standing cone[1–4]

A funnel with the end of the stem cut perpendicular to the axis of symmetry is secured with its tip a given height, H, above graph paper placed on a flat horizontal surface. Powder is carefully poured through the funnel until the apex of the conical pile so formed just reaches the tip of the funnel. The mean diameter, 2R, of the base of the powder cone is determined and the tangent of the angle of repose is given by $\tan \alpha = \frac{H}{R}$, where α = angle of repose.

Method II. Fixed Bed Cone[5]

The diameter of the base is fixed by using a circular dish with sharp edges, or a suitably machined container. Powder is poured on to the centre from a funnel which can be raised vertically until a maximum cone height, H, is obtained, $\tan \alpha$ being calculated as before.

Method III. Tilting Box[4]

A rectangular box is filled with powder and tipped until the contents begin to slide. The angle which the upper surface of the box makes with the horizontal is taken as the angle of repose.

Method IV. Revolving Cylinder[6–7]

A sealed hollow cylinder with one end transparent is made to revolve horizontally. It is half-filled with the powder, so that the free surface

of the powder forms a diametrical plane. The maximum angle that this plane makes with the horizontal on rotation of the container is taken as the angle of repose.

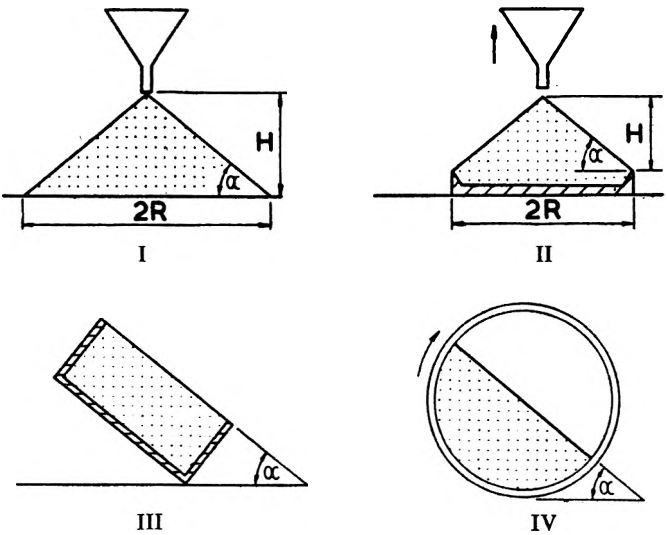

FIG. 1. Four main methods of measuring the angle of repose.
I. Fixed funnel and free standing cone.
II. Fixed bed cone.
III. Tilting box.
V. Revolving cylinder.

No published investigation has been found in which the methods are simultaneously compared, yet an inspection of reported results indicates a range in the angles of repose for what would appear to be the same material, e.g., lead shot, silver sand, or mustard seed. A critical examination of the methods indicated in Figure 1 has been made as a possible means of assessing this property.

EXPERIMENTAL AND RESULTS

All materials were graded by sieving in order to reduce complications introduced by separation during the practical runs. The size ranges of the powders used are reported in terms of the numbers of mesh of the B.S. sieves[8] firstly through which the particles will pass and secondly on which they will be retained (e.g., 60/80 powder). Simple free flowing materials were used in this series of experiments to ensure that complications due to shape and surface characteristics of the particles would be reduced to a minimum. The materials were allowed to reach equilibrium under conditions of 68° F. and 40 per cent relative humidity before the experiments were carried out.

Glass balls and lead shot. These two materials were chosen because they have a similar shape but differing specific gravities (glass, 2·2; lead, 11·3) and surface friction characteristics.

Sand. This was chosen because it had a characteristic shape, and a wide range of sizes were easily obtainable. Silver sand was wet sieved, dried and resieved to ensure reproducible grading.

Test of Method I. The technique described by Neumann[2] was followed using a funnel with a stem bore of 0·3 cm. diameter with the tip of the stem 2·0 cm. above the surface. But, it was noticed that the heap often collapsed before the top of the cone reached the tip of the funnel; this sort of slip gave an erroneously large value for the base diameter and hence a low value for the angle of repose. The incidence of experiments in which the heap collapsed is greater if the diameter of the stem bore is increased and, with any given stem diameter, if the rate at which powder is added to the heap is increased. With the largest particles a shallow cone resulted and this led to an investigation of the effect of varying the height of the stem tip above the horizontal surface. Experiments were also made in which the particles were not added to the heap using the funnel, but were added through a glass tube, of which one end was drawn out to a narrow tip, held at a shallow angle so that the flow of particles was under complete control. A selection of results is given in Table I.

TABLE I

MAXIMUM ANGLE OF REPOSE MEASUREMENTS USING A FUNNEL AT A FIXED HEIGHT ABOVE SURFACE

Material	Glass spheres		Lead shot		Silver sand	
Grading, mesh ..	100/120		8/10		60/80	
Conditions	A	B	A	B	A	B
Height of cone apex above surface, cm.	Tangent of angle of repose $\times 10^3$					
1·0	580	630	520	540	880	960
2·0	550	605	500	580	830	930
3·0	500	550	490	545	790	800
4·0	465	520	465	520	730	770
5·0	465	512	450	515	690	760
6·0	435	510	420	510	650	690

A. Maximum result obtained using Neumann's technique.
B. Maximum result obtained by careful addition of powder through a narrow inclined tube.

Column A represents the maximum result when the powder was poured through a funnel following Neumann's technique; column B represents the maximum result when all precautions are made to reduce particle momentum to a minimum. It was noticed with all materials that there was an occasional collapse of the heap apparently due to the material slipping on the paper surface. This effect was considerably reduced by using a horizontal surface of coarse sand paper but its use made the measurement of the base diameter of the heap difficult.

Test of Method II. Nelson's technique[5] was followed initially, but the momentum with which the particles hit the pile greatly increased the incidence of heap collapse. This observation led to an investigation of the best methods by which a cone could be made by carefully building up the pile by the technique described above. The results in Table II indicate the range which can be achieved between the published technique

(column A) and the alternative method (column B). The effect of varying the diameter of the base was investigated, and also, when the heap collapsed, the angle of repose of the surface of the remaining slope was measured (column C).

TABLE II

MAXIMUM ANGLE OF REPOSE MEASUREMENTS USING FIXED BED METHOD

Material	Glass spheres			Lead shot			Silver sand		
Grading, mesh	14/16			16/22			16/22		
Conditions	A	B	C	A	B	C	A	B	C
Radius of base, cm.	Tangent of angle of repose $\times 10^3$								
0·64	560	650	—	560	630	—	840	960	—
1·28	558	597	480	560	600	460	800	920	700
1·80	522	557	465	560	580	480	785	870	650
2·48	517	544	445	530	560	445	775	835	650
3·43	490	554	425	510	550	430	770	810	630
4·38	485	520	420	500	530	420	740	780	660
7·01	485	515	425	490	510	415	725	750	650
11·00	460	520	430	470	510	420	690	710	650

A. Maximum result using Nelson's technique.
B. Maximum result obtained by careful addition of powder through a narrow inclined tube.
C. Residual slope of heap measured after slip had taken place.

Test of Method III. Takahasi[4] did not indicate the size of box he used, but preliminary experiments using a 30/44 mesh grading of glass balls and various shaped boxes at hand indicated that the dimensions of the bed were of critical importance. The use of boxes was not satisfactory and a tilting table was devised (Figure 2) consisting of a flat plate (N) mounted

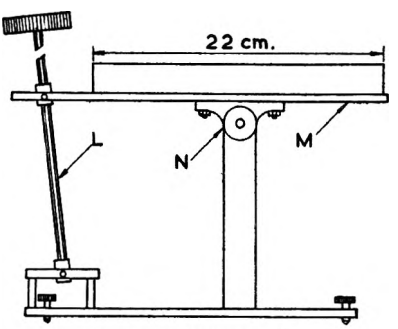

FIG. 2. Tilting table.

on standard pulley blocks (M) so that it was free to turn about a horizontal axis. Side pieces were mounted to this base plate to form the equivalent of three sides of a box. Movement was precisely controlled by the screwed rod (L). In use it was found that, to reduce base and wall effects, the depth of the bed should be at least 20 particle diameters of mean particle size (2 cm. depth is suitable for powders) and the width should be not less than one-third of the length of the bed. It was also found that a layer of coarse sand paper along the bottom of the bed was necessary to prevent preferential slip of the particles along this plane. The material was placed as a bed of given length and the excess cleaned

off to give a surface flush with the top of the table frame. The bed was then carefully tilted until slip occurred. The limiting angle at which this occurred was taken as the angle of repose. A selection of results is given in Table III.

TABLE III

MAXIMUM ANGLE OF REPOSE USING TILTING TABLE

Material	Glass spheres		Lead shot		Silver sand	
Grading, mesh ..	100/120	14/16	16/22	8/10	100/120	16/22
Length of bed, cm.	Tangent of angle of repose $\times 10^3$					
1·0	740	—	680	—	1010	—
2·0	760	780	720	—	960	880
3·0	680	640	660	620	920	940
5·0	630	650	620	630	850	830
7·0	615	625	630	630	780	800
10·0	580	600	570	595	760	760
14·0	570	570	550	560	740	725
18·0	550	550	570	550	725	730
22·0	560	540	550	540	715	725

Test of Method IV. Cylinders of various diameters were used. The curved walls were lined with sand paper in order to prevent preferential slip between the powder and the walls of the container. A selection of results is given in Table IV.

TABLE IV

MAXIMUM ANGLE OF REPOSE USING REVOLVING CYLINDER

Material	Glass spheres		Lead shot		Silver sand	
Grading, mesh ..	100/120	14/16	16/22	8/10	100/120	16/22
Diameter of cylinder, cm.	Tangent of angle of repose $\times 10^3$					
2·0	750	760	740	660	900	920
5·5	615	640	615	630	800	780
9·0	590	580	560	585	760	740
12·0	570	580	550	570	725	735

DISCUSSION

Inspection of the results indicates that, for any given method, the magnitude of the angle of repose falls as the size of the heap or bed increases. Direct comparison between methods is difficult because the basis for measurement of the size of the powder heap or bed varies from one method to another, although it is clear that methods I and II give results that are lower than those given by methods III and IV. This difficulty in comparison has been overcome by presenting the results in graphical form as in Figures 3 and 4. In these graphs the abscissa represents the horizontal base and a line joining a given point and the origin makes the angle of repose with this datum line.

All the results in each graph lie within a well defined zone, the lower boundary of which is the locus of the points recording the natural angle of slip (the results in Table II, column C) and the upper boundary of which is the locus of points obtained when a flat surface of the material is tilted until it does slip. These boundaries tend to straight lines for

larger measurements, but extrapolation of the straight portion of each line does not pass through the origin.

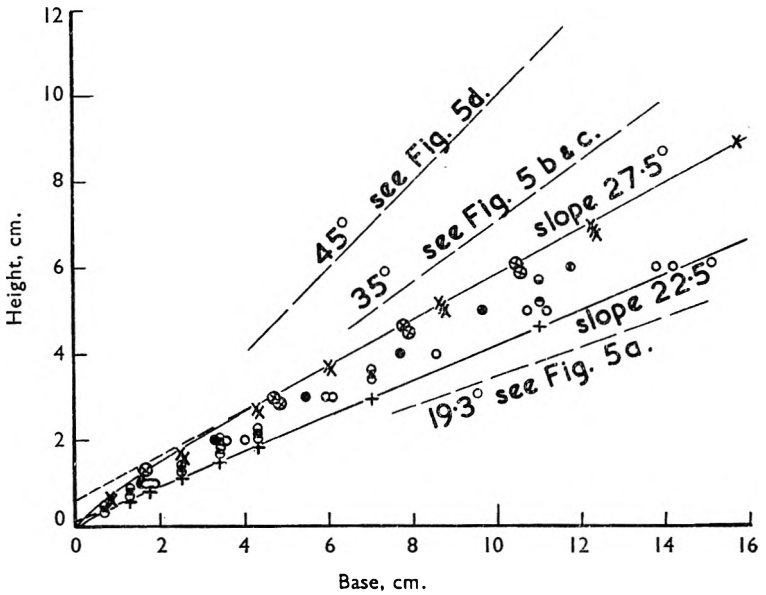

FIG. 3. Combined results for glass balls and lead shot of experimental angle of repose measurements. Key below Fig. 4.

For friction between flat surfaces, it is well established that the coefficient of static friction has a larger value than the coefficient of kinetic friction. With the present data, the angle of the slope of the linear portion of the upper boundary line represents the static angle of repose whilst the angle of the slope of the lower boundary line represents the dynamic angle of repose. This is in agreement with the observations of Franklin and Johanson[6] and of Pridham[7].

In practice, the position which an experimental result will occupy between the boundaries will depend on the technique adopted to measure the angle of repose, but all the data of this work (only a representative selection has been included in the Tables) confirm that results for a given material using a set procedure are reproducible. With the heaped cone techniques, the magnitude of the final ratio of height to base depends on reducing the momentum of the particles (otherwise the stability of the existing heap is upset and general slip takes place) and also in forming the heaps in such a way that in *all* sectors the slope is built up to the limiting angle (which, however, must not be exceeded, otherwise premature slip takes place). These are practical difficulties and, consequently, results tend to be low.

Figure 4 gives the results for silver sand. No size effect was noted except that for small heaps or beds, wall and end effects gave erratic results.

All the results for spheres have been included in Figure 3 irrespective of size or type of material. It was observed that failure in the case of an

assemblage of equally sized spheres of a given material was mainly by rolling of the added particles down the slope of the heap. This led to a

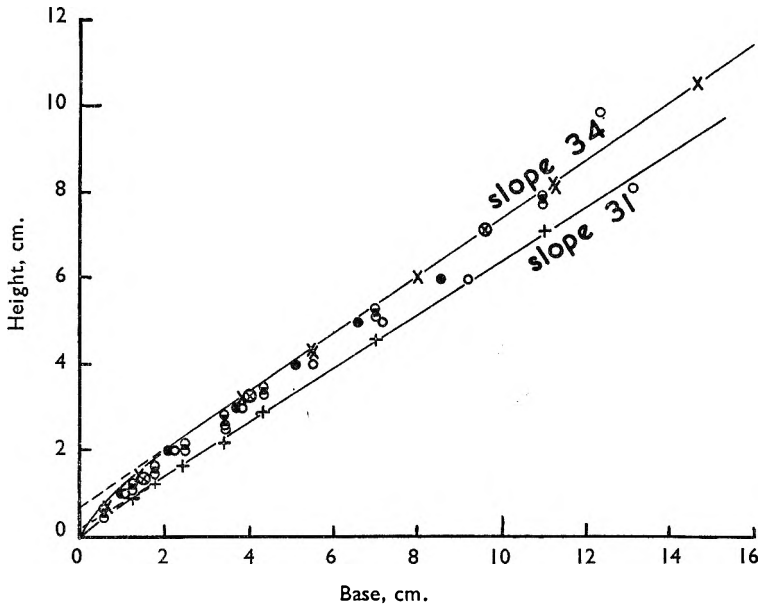

FIG. 4. Results for angle of repose measurements for silver sand.

Key to Figs. 3 and 4.

- ○ Method I, Neumann's technique (Table I, A).
- ● Method I, using narrow inclined tube (Table I, B).
- ◓ Method II, Nelson's technique (Table II, A).
- ◒ Method II, using narrow inclined tube (Table II, B).
- \+ Method II, Residual slope of heap after slip (Table II, C).
- × Method III, Tilting table (Table III).
- ⊗ Method IV, Revolving cylinder (Table IV).

consideration of the condition which would allow a particle to roll down the slope of randomly packed spheres of the same size, and it was found to depend mainly on the type of packing in the surface. A sphere will stay in a hollow formed by a grid of three or more spheres on a slope providing its centre of gravity does not fall outside the lower boundary of the grid. The closest packing on a regular triangular grid permits rolling if the slope of the plane is 19·3° ($\tan^{-1}$ 0·351) for one axis of symmetry (Figure 5*a*) and 35·1° ($\tan^{-1}$ 0·702) for the alternative axis (Figure 5*b*). In the case of regular square packing, the necessary slopes to permit rolling are 35·3° ($\tan^{-1}$ 0·709, Figure 5*c*) and 45° ($\tan^{-1}$ 1·0, Figure 5*d*). Thus for regular packings, the smallest angle for a slope on which rolling will take place is 19·3° and the largest possible angle which can prevent movement is 45°. Plane slopes were made using in turn samples of glass spheres, lead shot, $\frac{1}{4}$ in. steel ball bearings, and also table-tennis balls and in all cases these limiting conditions were confirmed. In practice, however, a surface of a heap will consist of a random selection of these grids and the

slope will have an angle similar to those given in Figure 3 where the slope of the lower boundary is 22·5° ($\tan^{-1}$ 0·414) and of the upper boundary is 27·5° ($\tan^{-1}$ 0·521).

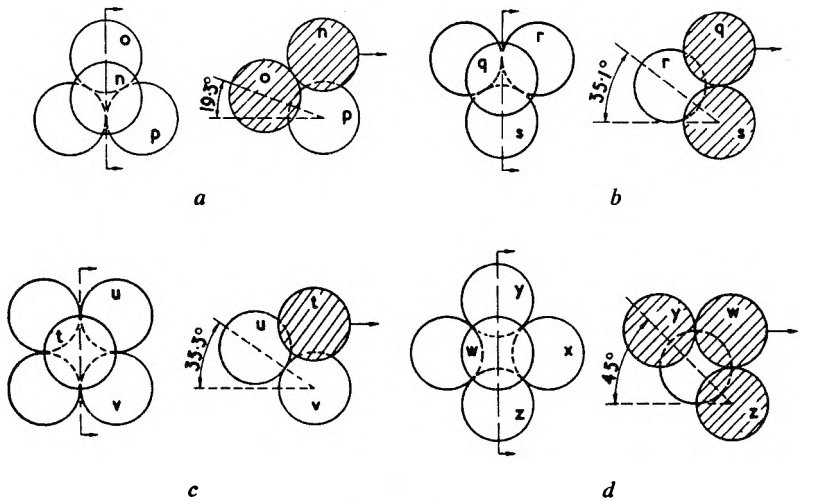

FIG. 5. Limiting conditions for rolling of a ball supported on sloping grids of 3 or 4 balls. See text for further explanation.

Recently, Brown and Richards[9] presented a paper to the Institution of Chemical Engineers describing yet another variation of angle of repose measurement. A circular platform of known diameter was immersed in the centre of a large container filled with the particulate material, which was allowed to escape slowly from the bottom of the container. The height of the pile remaining on the platform was measured to calculate the angle of repose. It is interesting that under these conditions the angle of repose for glass balls is reported as 18·9° and 20·7°. The method of Brown and Richards probably simulates the best conditions to measure interparticulate friction within a *moving* bed of particles and therefore, on the findings of my work, measures the dynamic angle of repose corresponding to the lower boundary slope in Figure 3.

CONCLUSION

Most methods of angle of repose measurements will provide the necessary data to allow suitable comparison between samples during routine quality control tests. However, in order to ensure reproducibility between workers or laboratories it is necessary to define rigid practical conditions.

Acknowledgement. I wish to thank Professor M. B. Donald and Mr. R. L. Brown for permission to refer to work[7,9] which is not yet published, to Mr. J. J. Deer for technical assistance in making the apparatus, and to Professor E. Shotton for his continued good counsel.

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THE FLOW PROPERTIES OF POWDERS UNDER HUMID CONDITIONS

By D. J. Craik and B. F. Miller

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The flow properties of different types of powder, with varying moisture contents, and the effects of adding small quantities of magnesium oxide, were measured. An explanation of the results in terms of adhesion by intermolecular forces and by the surface tension of moisture films is suggested.

The flow properties of starch powders, and of mixtures of starch with light magnesium oxide and other very finely divided powders, were recently investigated using the angle of repose method[1]. The electron microscope demonstrated the adsorption of the very fine powders on to the surfaces of the larger particles and it was considered that the greatly improved flow properties of the mixtures were due to the reduction of the adhesion between the particles. These investigations were made in the conditions prevailing in the laboratory and no account was taken of the moisture content of the starch.

A high moisture content is known to reduce the ease of flow of some powders, an effect which may be very pronounced with soluble crystalline powders. Experiments were designed, therefore, to measure the flow properties of powders with varying moisture contents, and to determine to what extent the addition of magnesium oxide might affect the properties under these conditions. Some commercial preparations contain additives presumably intended to counteract the effects of an appreciable moisture content on the ease of flow.

Experimental

Measurement of the angle of repose of a powder gives a reproducible numerical value for the flow properties so long as the conditions of the measurement remain constant. In these experiments the angle of repose was measured by pouring the powder through a funnel on to a horizontal sheet of graph paper, until the tip of the heap so formed reached the stem of the funnel. The diameter of the base was then read and the angle of slope of the side of the cone calculated. The end of the stem of the funnel was ground flat and was 3 mm. in diameter and 1·5 cm. above the plane of the paper. The accuracy of the measurement depends upon the kind of material measured. When the angle of repose is low the values are reproducible within 5 per cent, but when the angle is very large an irregular heap is formed and the variation of subsequent readings may be as much as 10 to 20 per cent.

Three very different kinds of material were examined; an organic amorphous powder, maize starch, an organic crystalline powder, sucrose, and an inorganic crystalline powder, sodium chloride. The samples of

sucrose and of sodium chloride were each of two different particle diameters of approximately 100 μ and 10 μ, called "coarse" and "fine", making a total of five samples. Six batches of 5 g. of each were prepared and 0, 0·25, 0·5, 1·0, 2·0 and 4·0 per cent of light magnesium oxide were added to each.

To obtain different values of the moisture content, the powders were placed in open jars, 4 cm. in diameter, in which they formed a layer less than 1 cm. deep, in a cabinet in which a controlled temperature and humidity could be maintained. The air in the cabinet was constantly circulated and from the work of Browne[2] on sucrose, and of Yee[3] on sodium nitrate, it was to be expected that the crystalline powders would attain equilibrium with the atmosphere in one, or two hours. Twenty-four hours was chosen as a convenient interval for exposure to any one humidity. Accurately weighed quantities of the five powders were also placed in open weighing bottles in the cabinet, and these were weighed whenever the angle of repose was measured so as to infer the moisture content of the samples. Weighing the samples on which the measurements were made would not have been significant owing to the slight loss of material incurred. The depth of the powder in the weighing bottles was equal to that in the jars.

The humidity in the cabinet was controlled by phosphorus pentoxide or saturated salt solutions[4], and measured by the dew point method; the temperature was maintained at 30°C. The cabinet was equipped with a Perspex front through which rubber gloves were sealed so that the angle of repose could be measured without removing the powders from the appropriate atmosphere.

Results

For the sake of clarity only those results obtained at four well-spaced humidity values will be considered, at 0 (nominally, actually less than 4 per cent), 42, 65, and 81 per cent relative humidity (R.H.). Results obtained at other humidities generally fitted in well between these. At 42 and 65 per cent R.H. the sugar and salt appeared to achieve equilibrium with the atmosphere within 24 hours shown by weighing the controls at intermediate times. At 81 per cent R.H. equilibrium had not been attained but measurements were taken at the end of 24 hours after which the powders continued to absorb moisture until they became too moist for measurement. At humidities above 81 per cent both the soluble powders deliquesced rapidly. In Table I the moisture contents are

TABLE I

MOISTURE CONTENT (PER CENT) AFTER 24 HOURS EXPOSURE TO THE HUMIDITIES INDICATED. THE VALUES CORRESPONDING TO 81 PER CENT R.H. DO NOT REPRESENT EQUILIBRIUM

Humidity (per cent)	Starch (per cent)	Sucrose 100 μ (per cent)	Sucrose 10 μ (per cent)	NaCl 100 μ (per cent)	NaCl 10 μ (per cent)
42	5·5	0·03	0·07	0·01	0·03
65	8·6	0·08	0·14	0·03	0·06
81	10·7	0·12	0·20	0·09	0·14

expressed as percentages on the assumption that the powders were dry after 60 hours over phosphorus pentoxide.

Graphs of the angle of repose against the concentration of added magnesium oxide for the five powders at four different humidity values, and corresponding water contents, are shown in Figures 1 to 5. Each set of curves shows different characteristics. The curve for starch at 42 per cent R.H. (Fig. 1) is similar to that previously obtained[1]. The effect of the variation of moisture content is regular over the whole range of concentrations, the shape of the curves not being appreciably altered.

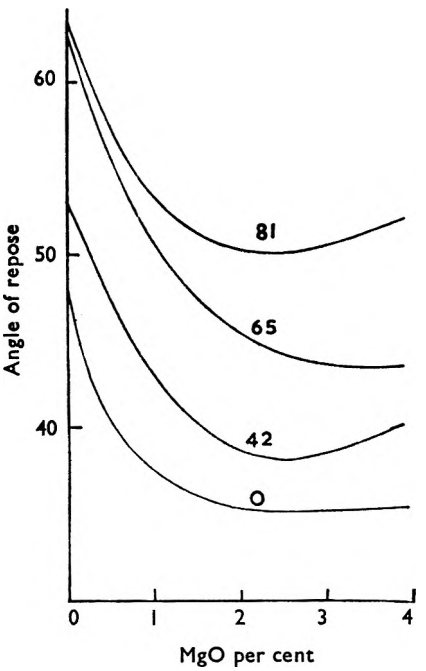

FIG. 1. The angle of repose of starch and mixtures with light magnesium oxide after 24 hours exposure to atmospheres of 0, 42, 65 and 81 per cent humidity at 30°.

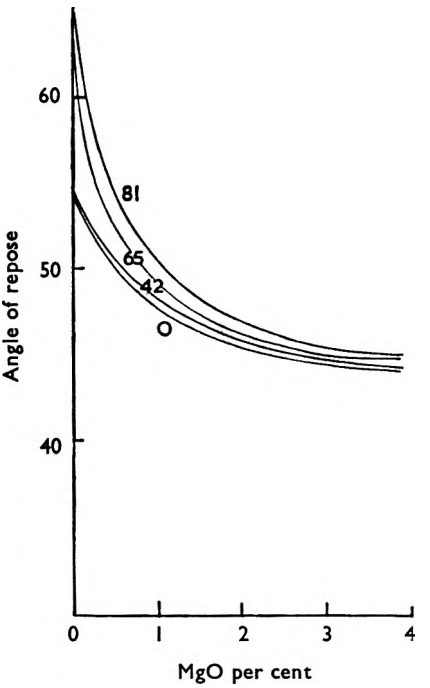

FIG. 2. The angle of repose of sucrose (10 μ) and mixtures with magnesium oxide at relative humidities of 0, 42, 65 and 81 per cent.

The graphs for the fine sugar (Fig. 2) show that at moderate humidities the effect of the added magnesium oxide is similar to that for starch, the angle of repose being reduced by approximately 12° for the 1 to 2 per cent mixture. When the humidity is raised the angle of repose of the fine sugar alone is increased but the mixture flows nearly as easily as before. The close spacing of the curves above 0·5 per cent magnesium oxide contrasts with those for starch.

The main difference in the behaviour of the coarse sugar (Fig. 3), compared with that above, is that when it is dry or at intermediate humidities (42 per cent), the addition of magnesium oxide does not reduce the angle and may even increase it slightly. When the humidity is raised to

81 per cent, however, and the moisture content is 0·12 per cent, the coarse sugar mixtures show the most striking effect of all, the angle being reduced from a high value to that typical of the dry powder.

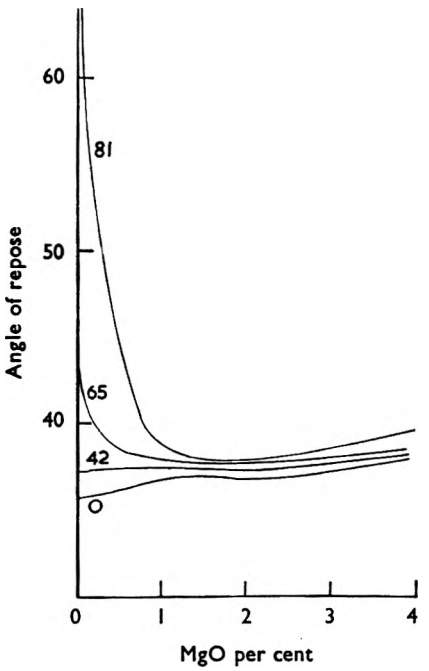

FIG. 3. The angle of repose of sucrose (100 μ) and mixtures with magnesium oxide at relative humidities of 0, 42, 65 and 81 per cent.

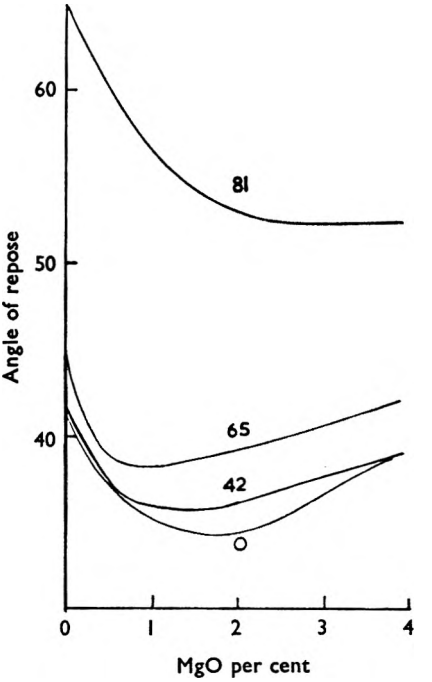

FIG. 4. The angle of repose of sodium chloride (10 μ) and mixtures with magnesium oxide at relative humidities of 0, 42, 65 and 81 per cent.

At low humidities the fine salt is affected in a similar way (Fig. 4), but to a lesser extent than the starch. At high humidities (81 per cent), the effect becomes much more pronounced but, unlike the fine sugar, the flow properties are not completely restored, this curve being well above the others.

The effect of the varying humidity on the coarse salt is similar to that for the coarse sugar (Fig. 5). The added magnesium oxide has a negligible effect on the angle of repose at low humidities but a great effect at higher humidities. The difference in appearance was striking in these conditions, the normal material being aggregated in large irregular clumps and sticking to the sides of the jar while the mixture generally appeared identical with the dry material.

Whether the powders had become caked on exposure to the various humidities was noted. The starch was only slightly caked at the highest humidities and the caking was suppressed by the addition of 0·25 to 0·5 per cent of magnesium oxide. The fine sugar caked considerably at 81, 70 and 65 per cent R.H. The caking was suppressed by the admixture of 1 per cent magnesium oxide at 81 per cent R.H., 0·5 per cent magnesium oxide at 70 per cent R.H., and 0·25 per cent magnesium oxide at

65 per cent R.H. The coarse sugar also caked at high humidities and this was suppressed by 0·5 per cent magnesium oxide at 81 per cent R.H., and 0·25 per cent magnesium oxide at 70 per cent R.H. Similarly, caking of the fine salt was suppressed by 0·5 per cent magnesium oxide at 81 per cent and 70 per cent R.H., and of the coarse salt by 0·25 per cent magnesium oxide at 70 per cent and 81 per cent R.H. For the purpose of these observations caking was considered to have occurred when the powder could not be moved by vigorously shaking or tapping the jar.

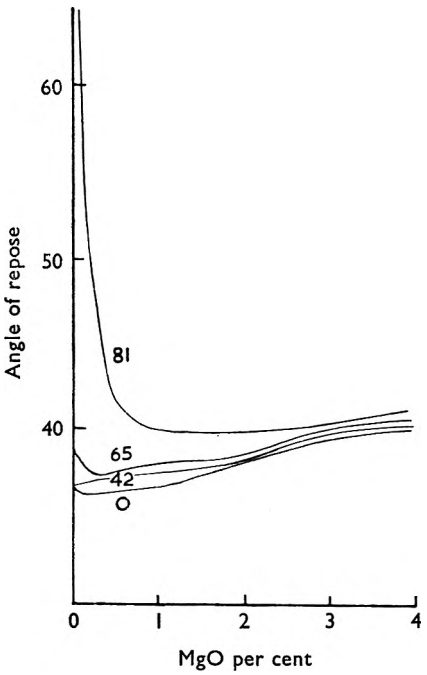

FIG. 5. The angle of repose of sodium chloride (100 μ) and mixtures with magnesium oxide at relative humidities of 0, 42, 65 and 81 per cent.

In addition to the effects on the flow properties of the powders, the addition of the magnesium oxide substantially diminishes the tendency to set or cake, more of the additive being necessary to achieve this result at higher humidities, and at the lower particle sizes.

Replicas, for examination in the electron microscope, of samples of the sugar and salt, and mixtures with magnesium oxide, were prepared by evaporating carbon on to the particles on a Formvar film and subsequently dissolving away the film and the particles. It was necessary to keep the sugar samples a considerable distance from the carbon rods to avoid melting. The carbon film containing the particles was floated on the surface of distilled water for about an hour to remove the salt or sugar. This treatment did not remove the magnesium oxide which remained attached to the carbon replica. The magnesium oxide was adsorbed on the surfaces of the sugar and salt particles to a considerable extent. When the concentration was low all the magnesium oxide was adsorbed, none being found on the intervening areas of the film. The surfaces of the crystals were never completely covered; when the concentration of the magnesium oxide was high, a large number of separate aggregates remained unadsorbed. The magnesium oxide was identified by taking selected area diffraction patterns.

DISCUSSION

Both sucrose and sodium chloride are deliquescent when exposed to humidities in excess of those at which the vapour pressure of the moisture in the atmosphere is equal to the vapour pressure of their saturated

solutions[5]. Thus, sodium chloride is deliquescent above 75 per cent R.H. at 30°C.[4], and sucrose above approximately 80 per cent R.H.[6] Below these points both these materials absorb very small amounts of moisture depending on the humidity, while above the critical points the moisture contents continue to increase with the time of exposure, no true equilibrium being attained until complete solution has occurred. This investigation, therefore, concerns the material either below the critical humidity, or a little above it before the absorption has proceeded very far. Starch is hygroscopic and absorbs much greater amounts of moisture at corresponding humidities with no tendency to go into solution.

To analyse the results, some quantitative relations concerning the angle of repose must be quoted, although a fuller discussion of the factors concerned and of the factors governing the flow of a powder must be reserved for a separate publication. The angle of repose corresponds qualitatively to the flow properties of a powder. A high angle (e.g., 50° to 60°) is obtained for powders which form large aggregates and can be caused to flow only with difficulty, while a low angle (30° to 40°) is obtained for powders which acquire a smooth surface and flow easily. Since the method gives a reproducible numerical value, it is reasonable to adopt it as a measurement of the flow properties.

It may be calculated that a powder consisting of spherical non-adhering particles will always have the same angle of repose, of approximately 30°, so long as the heap formed is stable. All particles tend to adhere, regardless of variable factors such as electrostatic charging[7], but the relative effect of this adhesion compared with the gravitational force on the particles, or to their momenta, depends upon the particle size. Using an approximate model it may be calculated that, for the materials here investigated, adhesion will be significant when the particle size is less than 100 μ. The source of this adhesion may be considered to lie in the short-range intermolecular forces which vary inversely with the seventh power of the separation of the molecules[8,9], while a theory of the attraction of macroscopic bodies, which did not make use of assumptions concerning the interaction of individual atoms or molecules, was developed by Lifshitz in terms of electromagnetic theory and this showed the force to be inversely proportional to the third or fourth power of the separation. When adhesion is significant the angle of repose is increased, and it may also be increased by extreme variation of the shape of the particles from the spherical, but this is not significant here.

The variation in the angle of repose of dry starch may then be considered to represent the conversion of an adhering powder to a non-adhering powder, by the presence of the adsorbed magnesium oxide. The adhesion is overcome because of the short range of the forces compared with the size of the magnesium oxide particles. The dry fine sugar is similarly affected by the addition of magnesium oxide, although the adhesion is not reduced to such a great extent by any concentration of the magnesium oxide. The particle sizes of both the starch and the fine sugar (approximately 10 μ) are well within the calculated limits for adhesion.

The particle sizes of the coarse sugar and salt are ten times as great and these are effectively non-adhering powders, when dry, having an angle of repose approaching 30°. It is thus to be expected that the angle of repose is not further reduced by the addition of magnesium oxide. When the moisture content is increased these powders show an increasing adhesion, which is largely overcome by small concentrations of magnesium oxide. The amount of water involved is very small.

These effects can be explained if it is assumed that the moisture is adsorbed as a surface layer on the crystals. The adhesional force, due to the surface tension in a liquid film, between two spherical particles can be calculated to be $2\pi a \left(\frac{2m}{300}\right)^{\frac{1}{2}}$ T dynes, where a is the particle radius (cm.), T is the surface tension of the liquid in dynes/cm. and the moisture content is m per cent. Comparing this with the gravitational force on the particles, the critical radius below which the particles adhere is approximately $500\,\mu$ when $m = 0{\cdot}1$ per cent. Since this is greater than the radius of any of the crystalline samples examined, this postulated liquid film introduces forces of sufficient magnitude to account for the adhesion.

The thickness of the moisture film, when $a = 100\,\mu$ and $m = 0{\cdot}1$ per cent is $0{\cdot}03\,\mu$. The particles of the magnesium oxide are approximately $0{\cdot}05\,\mu$ in diameter, and since the crystals are always separated by twice this distance, the moisture film will only be continuous over a few very small regions, and the forces due to surface tension will be greatly reduced.

Thus, if it can be assumed that the absorbed water resides on the surface of the crystal, a quantitative explanation can be given of both the effects of moisture on the flow properties of the crystalline powders, and the effects of the added magnesium oxide in maintaining the ease of flow of moist powders.

Appendix

To obtain independent evidence of the existence of the moisture films on which the above theory depends, the resistance of approximately 1 cm. cube of sodium chloride powder was measured and found to be 200, 120, 8, 4 and 2 megohms when the moisture content was 0, 0·02, 0·05, 0·1 and 0·2 per cent, respectively. If the moisture remained on the surface of the crystals a large drop in the resistance would be expected, since the conducting solution would carry the current. If the moisture entered the crystals, however, it would be necessary to assume that it could cause the formation of a large number of lattice vacancies, to increase the conductivity to such a great extent[11]. Since this would require a considerable amount of energy, it is unlikely to occur, and thus the conductivity measurements appear to verify the existence of the moisture films although they may not, at this stage, be regarded as a conclusive proof.

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DISCUSSION

The Papers were presented by DR. D. TRAIN and MR. D. J. CRAIK respectively.

THE CHAIRMAN. In Mr. Craik's paper, were the powders mixed with the magnesium oxide before placing in the humidity cabinets?

MR. N. J. VAN ABBÉ (Loughborough). Mr. Craik had found that there was a limiting concentration of magnesium oxide for optimal flow properties, but not so with talc, in contradiction to the work of Nelson. Could angle of repose indicate flow properties which would result in ready dispersibility in mixing?

MR. D. W. HUDSON (Hove). Would the results found explain the troublesome problem of layering seen with coloured talcs in filling machines? Was the specific gravity of the powder of importance?

MR. J. H. OAKLEY (London). Why did Dr. Train sieve his sand wet, dry it and then sieve it again? He could not correlate the two methods for obtaining angle of repose because Table I referred to 100/120 mesh and Table II to 14/16 mesh. What was the method of levelling, and how was the end point determined? Was the impacting a feature in the apparent divergence due to dimensions of the table?

MR. H. D. C. RAPSON (Betchworth). In Mr. Craik's paper, why did 10μ particles of sodium chloride appear to be less strongly adherent than 10μ particles of sucrose, which was contrary to argument based on van der Waal's forces. If sodium chloride were absolutely dry one would expect a resistance of 100 or 1000 times the 200 megohms reported for the apparently dry samples. The sodium chloride and sucrose were, therefore, not absolutely dry and the results might be explained by the film of surface water on the particles. Table I showed that the water content of the 10μ sucrose particles was higher than that of the sodium chloride and this would point to a slightly thicker water layer on the former and would probably explain its apparent higher adhesion in the angle of repose measurements. These water molecules between the surfaces must be considered in the interpretation of any adhesion between small particles.

DR. L. SAUNDERS (London). Was the surface tension mentioned on page *142 T* of Mr. Craik's paper that of a bulk salt solution?

DR. TRAIN replied. Nelson had used talc as an example of the effect of a lubricant on the angle of repose. He did not think that angle of repose would be a useful test to show efficiency of mixing in powders. Layering was associated with density as shown by Maítra and Coulson *Trans. Chem. Eng. Soc. Imp. Coll. Lond.*, 1948, **4**, 135. A shape factor was also

concerned. Wet sieving was more efficient than dry sieving and the final sieving was to insure that all aggregates had been broken up. Other size ranges than those shown in the Tables had been covered and there was no significant difference. The surface of the bed using a tilting table was standardised by sliding a steel rule from the right (Fig. 2); if pushed the other way, the powder tended to impact. In a small length bed the size of the particle which was just within and parallel to the surface was significant. This factor tended to have less effect as the bed length increased. The revolving cylinder was put in the jaws of a chuck on a lathe with a ruler aligned on the diametrical plane and both were turned together. When the bed collapsed the ruler was held stationary and this maximum angle achieved for this surface was checked by moving the cylinder again. The results were consistent and the method the easiest.

MR. CRAIK replied. The mixtures had been made up before exposure to the humid atmospheres. Water did not have considerable physical effect on the magnesium oxide particles since he postulated their effect to be purely as mechanical spacers. The authors were trying to measure the thickness of the water layers. A solid lubricant had a layer lattice structure which cleaved easily along the plane, and then slid easily. Magnesium oxide had a cubic crystal structure and would not cleave easily. A dye adsorbed on talc particles would effect the surface, and so the rate of flow. He could not explain the anomaly of the relatively free flow of sodium chloride which would almost certainly have a monolayer of water on the particles. Intermolecular forces would come into play whenever particles were closer than 0·1 μ. In some recently published work it was shown that when the separation increased to about 1 μ the attractive forces dropped off very rapidly and this fitted in with the separation of particles by a layer of magnesium oxide particles of 0·05 μ diameter. A layer of water molecules should make no significant difference to the intermolecular forces and any additional effect of the layer calculated from the surface tension would be infinitesimal.

OBSERVATIONS ON INCREASED DISINTEGRATION TIMES OF TABLETS ON STORAGE

By R. A. Ramsay

From the Pharmaceutical Development Division, CIBA Laboratories Limited, Horsham, Sussex

Received May 20, 1958

An attempt has been made to devise a simple and rapid method for determining whether tablets produced from a particular mass will show a significant increase in disintegration time on storage.

In the following experiments, an eight millimetre diameter punch on a single punch Comprex press was used. A sample of as soft a tablet as could possibly be handled was first made, followed by samples of tablets at gradually increasing pressures until the maximum pressure that could be applied by hand operating the machine was reached. The mass was hand filled into the die.

The "compression ratio"[1] was calculated by dividing the weight of the tablet in milligrammes by its height in millimetres. The disintegration

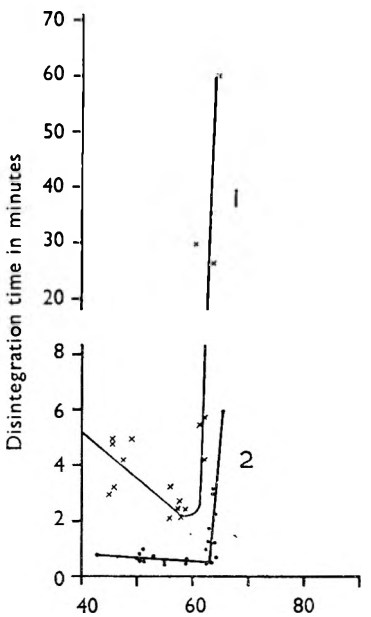

Fig. 1. The influence of "compression ratio" (weight/height) on the disintegration time of two formulations of glutethimide tablets (1) and (2), using an 8 mm. diameter punch.

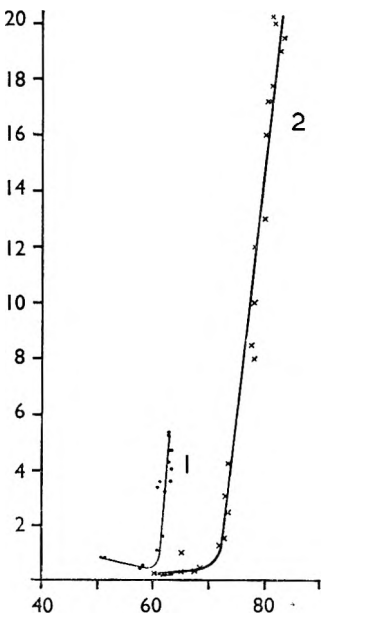

Fig. 2. The influence of "compression ratio" (weight/height) on the disintegration time of calcium thiocyanate nikethamide/theophylline tablets (1) and of phenyl (α-piperidyl) acetic acid methyl ester tablets (2) using an 8 mm. diameter punch.

time of the tablet in minutes was found by the B.P. method, one tablet at a time being tested. Graphs were prepared from the "compression ratio" and the disintegration time.

The results from the tablets made from a typical sample of a glutethimide mass are shown in Figure 1 (1). This mass contained 71 per cent glutethimide with gelatin and wheat starch as binders, wheat and arrowroot starches as disintegrating agents and magnesium stearate and talc as lubricants. Normal tablets made to this formula had an original disintegration time of under three minutes but after six months storage the disintegration time approximated to thirty minutes.

A typical sample of a second glutethimide mass was then examined. This mass also contained 71 per cent glutethimide and the same binders, disintegrating agents and lubricants, but 0·25 per cent of a wetting agent was added. The results are shown in Figure 1 (2). Normal tablets using this formula and process had shown no increase in disintegration time after two years storage.

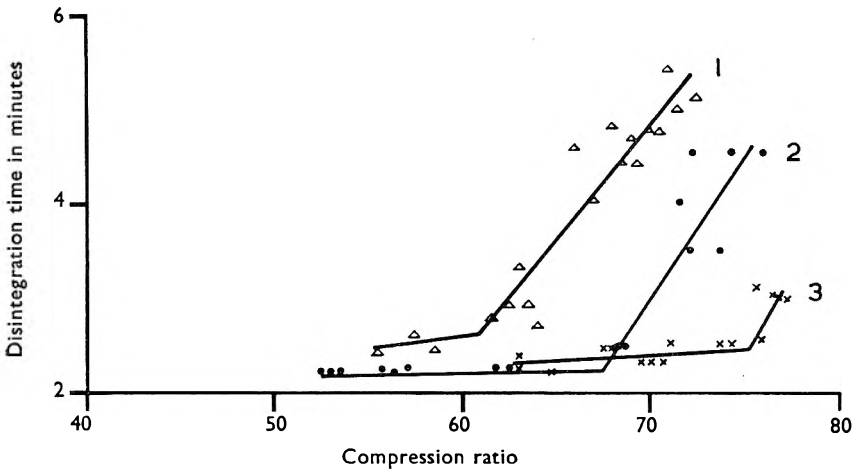

FIG. 3. The influence of "compression ratio" (weight/height) on the disintegration time of tolazoline tablets (1), of reserpine tablets (2) and of sulphathiazole tablets (3) using an 8 mm. diameter punch.

The graphs of "compression ratio" and disintegration time for freshly prepared tablets containing calcium thiocyanate, nikethamide, and theophylline, and of phenyl (α-piperidyl) acetic acid methyl ester are shown in Figure 2 (1 and 2). Normal tablets containing calcium thiocyanate, nikethamide, and theophylline gave an original disintegration time of thirty seconds which increased to fifteen minutes after three years storage, while tablets of phenyl (α-piperidyl) acetic acid methyl ester gave an original disintegration time of three minutes increasing to six minutes after three years storage.

The graphs of "compression ratio" to disintegration time for freshly prepared tablets of tolazoline hydrochloride, reserpine and sulphathiazole are shown in Figure 3 (1, 2 and 3). Normal tablets of these products gave no increase in disintegration time after three years storage.

For each tablet mass tested there exists a critical tabletting pressure. When this pressure is exceeded, the disintegration time of the tablets is increased. This phenomenon was illustrated by Berry and Ridout[1] who studied phenacetin tablets with 15 per cent potato starch and phenacetin tablets with various proportions of alginic acid as disintegrant, and also by Higuchi and others[2] in their studies on aspirin, lactose, lactose-aspirin and sulphadiazine tablets.

The graphs may be used to indicate whether tablets produced from a particular mass will show a significant increase in disintegration time on storage.

Comparing the graphs of the "compression ratio" to disintegration time for the two sets of glutethimide tablets, the main differences are (1) the variation in the size of the angle formed by the two approximately straight lines making up each graph and (2) the pronounced difference in the disintegration time of the tablets made under the maximum compressional force applied. The disintegration time of normal tablets from the first mass increased from under three minutes when freshly prepared to approximately thirty minutes after six months storage, whereas the disintegration time of the tablets made from the second mass remained unchanged after two years storage. Therefore, the greater the value of this angle and the lower the initial disintegration time at maximum compression, the less the risk of obtaining tablets with increased disintegration time on storage.

The graphs of the "compression ratio" to disintegration time for the other products tested show that the calcium thiocyanate, nikethamide and theophylline, tablet graph and the phenyl (α-piperidyl) acetic acid methyl ester tablet graph exhibit relatively acute angles in comparison to the angle exhibited by either the tolazoline hydrochloride, reserpine or sulphathiazole tablets graphs. The suggestion that the smaller the angle formed by the lines making up each graph, the greater the risk of obtaining increased disintegration times of the tablets on storage applies to the products investigated.

It will be necessary to study a much wider range of products and formulations to prove that these observations are applicable in all cases.

REFERENCES

1. Berry and Ridout, *J. Pharm. Pharmacol.*, 1950, **2**, 619.
2. Higuchi, Elowe and Busse, *J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 688.

DISCUSSION

The Short Communication was presented by the AUTHOR.

THE CHAIRMAN. Had the Author any views about the mechanism responsible for the increasing disintegration time on storage? Did the angle between the two portions of the graph take into account all the factors?

MR. D. STEPHENSON (Dartford). The Author had recorded results with and without a wetting agent. Had the addition of the wetting agent

in a volatile solvent in which the glutethimide ingredients were insoluble been tried, and had the results been compared with a granulation in which the wetting agent had been included from the beginning?

DR. D. TRAIN (London). Was there any evidence that the 0·25 per cent of wetting agent affected the strength of the tablet or resistance to abrasion?

MR. H. S. BRAGG (Folkstone). Could details of the weight and thickness of the tablets be given? Hand manipulation might give a very hard tablet which would not be obtained in practice.

MR. G. R. WILKINSON (London). Had an attempt been made to measure the pressure in the tabletting machine used?

PROFESSOR E. SHOTTON (London). The compression ratio did not necessarily give a true measurement of the compression. Were the tablets of constant weight? The compression ratio was valid only with one set of punches and dies, and its effect would depend on whether the punches were flat or concave. Which were used?

MR. A. BRAGG (Liverpool). Were the granule size, fines content and moisture controlled? All these factors would affect the disintegration time and might have a significant effect on storage.

MR. ANDERSON (Liverpool). What were the storage conditions? What was the wetting agent used and why was it chosen?

MR. N. S. VAN ABBÉ (Loughborough). He had observed that fluctuating temperature in a sealed container could give a hardening effect, possibly due to redistribution of moisture and this, together with compression, might be the mechanism responsible for the effects.

THE AUTHOR replied. Increase in disintegration time was often experienced in tablets with a high proportion of active ingredient and with ingredients of low melting point and high solubility in water. The wetting agent had been added to the original mass and not to the granules. A wetting agent allowed water to penetrate the mass and the disintegration at maximum pressure was affected but there was no difference in hardness. The abnormal pressures were used only to see whether the formulation was suitable, normal pressure would give tablets on the horizontal part of the graph. They had no means of measuring the pressure and, therefore, used relative compression. The graphs could only be used for a particular set of dies and punches, and those used were flat. The tablets were 200 mg. $\pm$ 10 per cent. They had tried to keep the method of production of the granules the same; no analysis of granule size had been made. The moisture content was probably a significant factor in the increased disintegration time on storage. Normal laboratory conditions were used in storage. The wetting agent was a sodium laurylsulphate.

THE PREPARATION AND THE ANTIBACTERIAL AND ANTIFUNGAL PROPERTIES OF SOME SUBSTITUTED BENZYL ALCOHOLS

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A number of substituted benzyl alcohols have been prepared and together with some related, commercially available, compounds have been tested for antibacterial and antifungal properties. The most active inhibitory compound was 3:4:5-trichlorobenzyl alcohol followed by 4-chloro-3:5-dimethyl-, 3:4-dichloro- and 2:4-dichlorobenzyl alcohols, but saturated aqueus solutions of the last three compounds were more rapidly bactericidal. Pharmacological tests have shown that 2:4-dichlorobenzyl alcohol has low toxicity.

Antibacterial properties were first attributed to benzyl alcohol by Nördlinger[1] in 1915. A further study by Macht and Satani[2] showed that a 0·2 per cent aqueous solution was bacteriostatic to *Staphylococcus aureus* and *Escherichia coli* and a 3 per cent solution was lethal to these organisms in 10 minutes.

Benzyl alcohol also found some application in medicine for its local anaesthetic properties[3,4], and in recent years the alcohol has been used as a preservative. In 1952 Gershenfeld[5] indicated its value in parenteral solutions by showing that a 1 per cent aqueous solution was an effective bacteriostat against *Staph. aureus*, *E. coli*, *Bacillus subtilis*, *Bacillus mesentericus* and *Bacillus megatherium*. Further aspects on the use of benzyl alcohol as a preservative have been considered by Kleine, Millwood and Walther[6], and by Royce and Sykes[7].

Since halogenation of phenols produces compounds with enhanced antiseptic properties[8], we considered that chlorinated benzyl alcohols might possess improved antibacterial and antifungal activity. Preliminary experiments confirmed this and a wide range of substituted benzyl alcohols was prepared. These, together with other related, commercially available, compounds were tested against a variety of bacteria and fungi to evaluate their *in vitro* activities. Pharmacological toxicity tests were made on two of the more active compounds. When this work had been completed a war-time French patent[9] was found which attributed antiseptic properties to some substituted hydroxyalkyl- and hydroxyalkenylbenzenes.

Chemical

A series of substituted benzyl alcohols was prepared and experimental details are given for those compounds which have not previously been described in the literature.

2:4:5-*Trichlorobenzyl alcohol.* A mixture of 2:4:5-trichlorotoluene[10] (36·5 g., 0·186 mol.), sulphuryl chloride (25·25 g., 0·186 mol.), and

benzoyl peroxide (0·23 g.) (see Kharasch and Brown[11]) was heated under reflux for 3 hours and then distilled to give 2:4:5-trichlorobenzyl chloride (24·8 g., 58 per cent), b.p. 114 to 120°/4 to 5 mm. The chloride (30 g., 0·13 mol.) was stirred at 95° for 48 hours with a solution of potassium carbonate (13·8 g., 0·1 mol.) in water (100 ml.), the mixture cooled, and the product collected and crystallised from light petroleum (b.p. 60 to 80°) to give 2:4:5-*trichlorobenzyl alcohol* (11·9 g., 43 per cent), m.p. 111 to 113°. Found: C, 40·3; H, 2·3 per cent. $C_7H_5OCl_3$ requires C, 39·7; H, 2·4 per cent.

3:4:5-*Trichlorobenzyl alcohol.* Similar treatment of 3:4:5-trichlorotoluene[12] gave, via 3:4:5-trichlorobenzyl chloride, b.p. 121 to 124°/4·5 mm., 3:4:5-*trichlorobenzyl alcohol* which crystallised from light petroleum (b.p. 80 to 100°) as needles, m.p. 108 to 110°. Found: C, 39·7; H, 2·1 per cent.

2:3:*x*:*y*-*Tetrachlorobenzyl alcohol.* As described by Beilstein and Kuhlberg[13], benzyl chloride (233 g.) was chlorinated exhaustively in the presence of first iodine (1 g.) and then antimony trichloride (18 g.). Crystallisation from chloroform-methanol of the crude product gave 2:3:4:5:6-pentachlorobenzyl chloride (12 per cent), m.p. 98·5 to 100·5° (*idem*[13] give m.p. 103°). Using the method of Ross and Markarian[14] the chloride was converted into 2:3:4:5:6-pentachlorobenzyl alcohol, m.p. 194 to 196° (*idem*[14] give m.p. 197 to 198°). Distillation (15 cm. Hempel column) of the content of the above chloroform-methanol mother-liquor gave a fraction, b.p. 148 to 166°/4 mm., refractionated to give an oil, b.p. 120 to 130°/1·5 mm. Found: C, 32·0; H, 1·3 per cent. $C_7H_3Cl_5$ requires C, 31·8; H, 1·1 per cent. This oil (21·5 g.) was heated under reflux for 20 hours with anhydrous sodium acetate (26 g.) and glacial acetic acid (250 ml.), the mixture evaporated to 100 ml. and diluted with water (500 ml.). The oily acetate mixture (17·2 g.) was isolated with chloroform and then boiled under reflux for 1 hour with ethanolic sodium hydroxide (200 ml., 5 per cent). After evaporation to 100 ml., the mixture was diluted with water (300 ml.) and the gummy precipitate crystallised from chloroform to give the pentachlorobenzyl alcohol (1·75 g.). Repeated crystallisation from chloroform-light petroleum (b.p. 40 to 60°) of the content of the mother-liquor gave 2:3:*x*:*y*-*tetrachlorobenzyl alcohol* (0·5 g.) as needles, m.p. 128 to 130°. Found: C, 33·95; H, 1·8 per cent. $C_7H_4OCl_4$ requires C, 34·15; H, 1·6 per cent.

4-*Chloro*-3:5-*dimethylbenzyl alcohol.* 4-Chloro-3:5-dimethylbenzoic acid[15] (3·7 g., 0·02 mol.) was converted with boiling methanol (25 ml.) and concentrated sulphuric acid (0·68 ml.) (4 hours) into the methyl ester (3·8 g.) which was dissolved in dry ether (200 ml.) and added dropwise to a stirred mixture of lithium aluminium hydride (0·5 g., 0·013 mol.) in dry ether (150 ml.). After 2 hours the mixture was decomposed with dilute sulphuric acid and the ether-soluble product crystallised from light petroleum (b.p. 40 to 60°) to give the *alcohol* as needles (1·6 g., 47 per cent), m.p. 39 to 41°. Found: C, 63·6; H, 6·6 per cent. $C_9H_{11}OCl$ requires C, 63·4; H, 6·5 per cent.

4-*Iodo*-3:5-*dimethylbenzyl alcohol.* A mixture of 2-iodomesitylene[16] (78 g.) manganese dioxide[17] (27·8 g.), and 62 per cent sulphuric acid (96 ml.) was stirred at 65° for 8 hours. The cold mixture was diluted with water and the neutral product, isolated with ether, distilled to give 2-iodomesitylene (67 per cent recovery) and a semi-solid (6·9 g.), b.p. 120 to 160°/2 mm. The last was triturated with light petroleum (3 × 5 ml.) and the residue crystallised from methanol to give 2:4-diiodomesitylene as plates (1·1 g.), m.p. 83 to 84° (cf. Töhl and Eckel[18] who give m.p. 82 to 83°). Found: C, 29·4; H, 2·6; I, 67·7 per cent. Calc. for $C_9H_{10}I_2$: C, 29·0; H, 2·7; I, 68·3 per cent. Chromatography over alumina (100 g., Spence type "H") of the content of the light petroleum and methanol solutions gave a solid (3·1 g.) eluted with light petroleum (b.p. 40 to 60°) (500 ml.), which was crystallised first from methanol to give 2:4-diiodomesitylene (0·2 g.), and then from aqueous methanol to give 4-*iodo*-3:5-*dimethylbenzaldehyde* (1·85 g., 2 per cent) as needles, m.p. 65 to 67°. Found: C, 41·7; H, 3·6 per cent. $C_9H_{10}OI$ requires C, 41·5; H, 3·5 per cent. The 2:4-*dinitrophenylhydrazone* crystallised from chloroform as crimson needles, m.p. 283 to 284°. Found: N, 13·2 per cent. $C_{15}H_{13}O_4N_4I$ requires N, 12·7 per cent.

A solution of the aldehyde (3·7 g.) in methanol (20 ml.) was treated dropwise with a solution of potassium borohydride (0·2 g.) in water (5 ml.). After 2 hours, the mixture was evaporated under reduced pressure to 8 ml., cooled, acidified, diluted with water, and the product isolated with ether. Crystallisation from light petroleum (b.p. 40 to 60°) gave 4-*iodo*-3:5-*dimethylbenzyl alcohol* (2·2 g., 60 per cent) as elongated prisms, m.p. 82 to 83°. Found: C, 41·1; H, 4·2 per cent. $C_9H_{11}OI$ requires C, 41·2; H, 4·2 per cent.

6-*Nitropiperonyl alcohol.* A suspension of 6-nitropiperonal[19] (20 g., 0·1 mol.) in methanol (250 ml.) was stirred during the dropwise addition of potassium borohydride (2 g., 0·038 mol.) in water (20 ml.). After 1 hour, the solution was acidified and poured into water. Crystallisation of the precipitate from benzene gave the *alcohol* (17·4 g., 86 per cent) as pale yellow needles, m.p. 122 to 123°. Found: C, 48·5; H, 3·5; N, 7·3 per cent. $C_8H_7O_5N$ requires C, 48·7; H, 3·55; N, 7·1 per cent. Mono acetyl derivative: m.p. 149 to 150°. Found: C, 50·5; H, 3·7; N, 6·2 per cent. $C_{10}H_9O_6N$ requires C, 50·2; H, 3·8; N, 5·9 per cent.

2:4-*Dichlorobenzyl propionate**. The *ester*, b.p. 114°/1 mm., was prepared by treatment of 2:4-dichlorobenzyl alcohol (7·2 g.) with boiling propionic anhydride (9·5 ml.) and a trace of concentrated sulphuric acid for 4 hours. Found: C, 51·4; H, 4·4 per cent. $C_{10}H_{10}O_2Cl_2$ requires C, 51·5; H, 4·3 per cent.

2:4-*Dichlorobenzyl methyl ether.* A mixture of 2:4-dichlorobenzyl chloride (31·5 g., 0·161 mol.) and sodium methoxide (from 3·8 g., 0·165 g. atom sodium) in methanol (250 ml.) was heated under reflux for 6 hours, cooled, filtered, and evaporated. Partition of the residue between water

* Prepared by Mr. J. Fraser.

and ether and distillation of the content of the ether phase, gave 2:4-*dichlorobenzyl methyl ether* (18 g., 59 per cent), b.p. 73°/0·6 mm. Found: C, 50·2; H, 4·3 per cent. $C_8H_8OCl_2$ requires C, 50·3; H, 4·4 per cent.

Sodium benzyl sulphate. A solution of benzyl alcohol (5 g., 0·046 mol.) in dry pyridine (25 ml.) was treated with sulphamic acid (13·5 g., 0·138 mol.) and the mixture heated to 83°. When the exothermic reaction had abated, heating on the steam bath was continued for 15 minutes. The cold mixture was filtered, the filtrate evaporated under reduced pressure, and the residue treated with aqueous *N*-sodium hydroxide (46·3 ml., 1 equiv.). On evaporation, the salt separated; crystallisation from a little water gave *sodium benzyl sulphate* (2 g., 20 per cent) as plates, m.p. 208 to 210°. Found: C, 39·6; H, 3·3 per cent. $C_7H_7O_4SNa$ requires C, 40·0; H, 3·3 per cent.

Sodium 2:3:4:5:6-*pentachlorobenzyl sulphate monohydrate* was prepared in a similar way and crystallised from very dilute (pH 8) sodium hydroxide as elongated plates (41 per cent), m.p. 270 to 272° (slow rate of heating). Found, in a sample dried over phosphorus pentoxide in a vacuum at 22°: C, 21·6; H, 1·1; H_2O 4·7 per cent. $C_7H_2O_4SCl_5Na$, H_2O requires C, 21·0; H, 1·0; H_2O, 4·5 per cent.

Some of the remaining alcohols were obtained commercially; the others were prepared by known methods and their properties agreed with those cited in the literature.

Microbiological Evaluation

Inhibition tests were made on serial dilutions of all compounds at pH 4·5, 6·5, and 8·0 and lethal tests on unbuffered solutions of those compounds which showed good inhibitory activity. Where necessary the compound was first dissolved in alcohol such that the final concentration of alcohol did not exceed 2 per cent.

The inhibition tests were made by dissolving the test compound in 10 ml. amounts of a tryptic digest glucose broth diluted ten times with tap water and buffered to the requisite pH with a sodium phosphate, citric acid buffer. The organisms used were Gram-positive bacteria (G+B), Gram-negative bacteria (G−B) and mixed mould spores, each group being considered separately. For the tests with bacteria, 24 hour cultures in nutrient broth were used, equal volumes of each strain being mixed together just before use; the G+B comprised *Staph. aureus* (3 strains), *Staph. albus* and *Streptococcus faecalis* and the G−B were *Proteus vulgaris*, *E. coli* and several *Pseudomonas* cultures including ten strains of *Pseudomonas pyocyanea*. For the tests with mould spores, seven-day old cultures of species of *Aspergillus*, *Penicillium*, *Cladosporium* and *Mucor* grown on honey agar were gently scraped off and suspended in sterile water containing a small amount of wetting agent (1 in 1000 Solution of Sulphestol). After mixing and straining to remove any large clumps, this suspension, containing approximately 1×10^8 spores per ml., was used.

To each 10 ml. of the prepared dilution of the test compound, 0·05 ml. of the suspension of the appropriate organisms was added and after incubation for 5 days at 25° the results were noted. The minimum

inhibitory dilution was taken as that dilution which just prevented growth.

TABLE I

ANTIBACTERIAL AND ANTIFUNGAL PROPERTIES OF NUCLEAR-SUBSTITUTED BENZYL ALCOHOLS

Compound	Substituent group(s) introduced CH_2OH 2	3	4	5	6	Approx. solubility in water at 20° (1 in −)	Minimum inhibitory concentration at pH 6·5 (1 in −) G + B	G − B	Moulds
Benzyl alcohol	—	—	—	—	—	25	300	200	300
4-Chlorobenzyl alcohol	—	—	Cl	—	—	400	400	400	800
2:4-Dichlorobenzyl alcohol	Cl	—	Cl	—	—	1000	2000	4000	6600
3:4-Dichlorobenzyl alcohol	—	Cl	Cl	—	—	1250	4000	4000	6600
2:4:5-Trichlorobenzyl alcohol	Cl	—	Cl	Cl	—	6000	10,000	N.A.M.S.	10,000
3:4:5-Trichlorobenzyl alcohol	—	Cl	Cl	Cl	—	6000	20,000	10,000	20,000
2:3:x:y-Tetrachlorobenzyl alcohol	Cl	Cl	←	2Cl	→	15,000	20,000	N.A.M.S.	N.A.M.S.
Pentachlorobenzyl alcohol	Cl	Cl	Cl	Cl	Cl	500,000	N.A.M.S.	N.A.M.S.	N.A.M.S.
2-Bromobenzyl alcohol	Br	—	—	—	—	750	750	750	750
4-Bromobenzyl alcohol	—	—	Br	—	—	450	2000	1000	1000
4-Iodobenzyl alcohol	—	—	I	—	—	2400	2400	N.A.M.S.	2400
4-Methylbenzyl alcohol	—	—	CH_3	—	—	160	500	500	500
2:4-Dimethylbenzyl alcohol	CH_3	—	CH_3	—	—	220	1000	1000	1000
4-Chloro-3:5-dimethylbenzyl alcohol	—	CH_3	Cl	CH_3	—	1800	6600	4000	6600
4-Iodo-3:5-dimethylbenzyl alcohol	—	CH_3	I	CH_3	—	10,000	20,000	N.A.M.S.	10,000
2-Hydroxybenzyl alcohol	OH	—	—	—	—	15	500	200	200
3-Hydroxybenzyl alcohol	—	OH	—	—	—	15	200	200	200
4-Hydroxybenzyl alcohol	—	—	OH	—	—	150	200	200	200
Piperonyl alcohol	—	O—CH_2—O		—	—	350	N.A.M.S.	N.A.M.S.	N.A.M.S.
6-Nitropiperonyl alcohol	—	O—CH_2—O		—	NO_2	6000	6000	N.A.M.S.	N.A.M.S.
Vanillyl alcohol	—	OCH_3	OH	—	—	500	N.A.M.S.	N.A.M.S.	N.A.M.S.
Anisic alcohol	—	—	OCH_3	—	—	500	500	N.A.M.S.	N.A.M.S.
2-Nitrobenzyl alcohol	NO_2	—	—	—	—	200	2000	1000	2000
3-Nitrobenzyl alcohol	—	NO_2	—	—	—	1700	N.A.M.S.	N.A.M.S.	N.A.M.S.
4-Nitrobenzyl alcohol	—	—	NO_2	—	—	500	660	660	660
4-Cyanobenzyl alcohol	—	—	CN	—	—	30	350	350	350
4-Hydroxycarbonylbenzyl alcohol	—	—	CO_2H	—	—	100	500	350	100
2:4-Dimethyl-5-hydroxymethylbenzyl alcohol	CH_3	—	CH_3	CH_2OH	—	820	N.A.M.S.	N.A.M.S.	N.A.M.S.
Cumic alcohol	—	—	C_3H_7	—	—	8000	N.A.M.S.	N.A.M.S.	N.A.M.S.

N.A.M.S. = Not active at maximum solubility.

For the lethal tests, in which bacteria only were used, dilutions of the selected compounds were prepared in water and 0·1 ml. of a combined mixture of organisms, as used in the inhibition test, was added to 10 ml. of each dilution. After 15 minutes, and at intervals during 24 hours, 0·1 ml. amounts were transferred to tubes each containing 10 ml. of saline and 1 ml. from each tube was plated with nutrient agar. Counts of the surviving organisms were made after incubation at 37°. The lethal time for a particular concentration was taken as being the time at which no surviving organisms were found. This was equivalent to a kill of over 99·99 per cent.

Results

The results of the inhibitory tests and the approximate solubilities of the compounds in water at 20° are given in Tables I and II. The majority of compounds showed little difference in activity over the pH range tested and consequently results at pH 6·5 only are shown.

The results of the lethal tests are given in Table III.

Discussion of Microbiological Results

Table I summarises the bacterial and fungal inhibitory properties of the nuclear-substituted benzyl alcohols. Introduction of a hydroxyl, methylenedioxy, cyano, or carboxyl group caused little or no increase in activity, whilst an alkoxyl or hydroxymethyl group removed activity. Nitration had the greatest effect at the 2-position whilst the 3-compound was inactive.

The most effective compounds were the halogenated benzyl alcohols. As the number of chlorine substituents increased the activity rose until a maximum was reached with 3 : 4 : 5-trichlorobenzyl alcohol. A tetrachloro alcohol was active only against Gram-positive bacteria and the fully substituted alcohol was inactive. The decrease in activity of the last two compounds was attributed to their very low solubility in water. 4-Bromobenzyl alcohol was more effective than the 2-isomer and was also more active than the 4-chloro compound whilst 4-iodobenzyl alcohol was much less soluble and inactive against Gram-negative bacteria. A methyl substituent in the nucleus gave an alcohol of increased activity but a larger grouping such as *iso*propyl decreased the solubility and activity was lost. Berger, Hubbard and Ludwig[20] found that the most active members of a series of phenyl ethers of glycerol, propylene and trimethylene glycols possessed a 4-chloro-3 : 5-dimethyl substitution pattern (cf. chloroxylenol), but in the benzyl alcohol series the activity of the 4-chloro-3 : 5-dimethyl compound was only of the same order as that of the two dichloro-alcohols examined. The 4-iodo-3 : 5-dimethyl- compound was much less soluble and was inactive against Gram-negative bacteria.

The effect of side-chain substitution is summarised in Table II. Activity was destroyed by esterification, etherification or sulphation of the hydroxyl-group. Substitution at the carbon atom by methyl, carboxyl, alkoxycarbonyl, or hydroxymethyl groups produced no increase in activity.

One phenyl substituent produced marked inhibition against Gram-positive bacteria and moulds but the compound lacked effect on Gram-negative bacteria, whilst triphenyl carbinol was so insoluble as to be devoid of activity. The more soluble hydrochlorides of diphenyl (piperid-2-yl-)- and diphenyl (piperid-4-yl) methanols had low activity.

TABLE II

ANTIBACTERIAL AND ANTIFUNGAL PROPERTIES OF SIDE-CHAIN SUBSTITUTED BENZYL ALCOHOLS

Compound	Substituent group(s) introduced			Approx. solubility in water at 20° (1 in −)	Minimum inhibitory concentration at pH 6·5 (1 in −)		
					G + B	G − B	Moulds
Benzyl alcohol	—	—	—	25	300	200	300
Benzyl methyl ether	—	OCH_3	—	330	N.A.M.S.	N.A.M.S.	N.A.M.S.
Sodium benzyl sulphate	—	$NaSO_4$	—	6	<100	<100	<100
Sodium pentachlorobenzyl sulphate	—	$NaSO_4$	—	850	N.A.M.S.	N.A.M.S.	N.A.M.S.
2:4-Dichlorobenzyl propionate	—	$CH_3CH_2CO_2$	—	5000	N.A.M.S.	N.A.M.S.	N.A.M.S.
2:4-Dichlorobenzyl methyl ether	—	OCH_3	—	6000	N.A.M.S.	N.A.M.S.	N.A.M.S.
Phenylethylene glycol	CH_2OH	—	—	3·5	<100	<100	<100
α-Phenylethanol	CH_3	—	—	150	300	300	300
Diphenyl carbinol	C_6H_5	—	—	2000	2000	N.A.M.S.	2000
Triphenyl carbinol	C_6H_5	—	C_6H_5	100,000	N.A.M.S.	N.A.M.S.	N.A.M.S.
Mandelic acid	CO_2H	—	—	6	500	100	<100
Ethyl mandelate	$CO_2C_2H_5$	—	—	100	500	200	200
Benzilic acid	CO_2H	—	C_6H_5	250	500	250	N.A.M.S.
α-Phenyl*iso*propanol	CH_3	—	CH_3	200	200	200	200
Diphenyl(piperid-2-yl)methanol hydrochloride	C_6H_5	—	$C_6NH_{10}HCl$	20	100	200	100
Diphenyl(piperid-4-yl)methanol hydrochloride	C_6H_5	—	$C_6NH_{10}HCl$	80	350	350	200

N.A.M.S. = Not active at maximum solubility.

The results of the lethal tests with some of the more inhibitory compounds are summarised in Table III, and show that 2:4-dichloro-, 3:4-dichloro-, 4-chloro-3:5-dimethylbenzyl alcohols have pronounced lethal properties. 3:4:5-Trichlorobenzyl alcohol, which showed maximum activity in the inhibition test, was lethal in 24 hours only as a saturated solution.

PHARMACOLOGICAL STUDY OF SELECTED COMPOUNDS

Approximate lethal doses (LD50) in mice were determined on selected compounds and the results are shown in Table IV. The apparent decrease in toxicity of 2:4-dichlorobenzyl alcohol on subcutaneous injection is under investigation.

A more detailed toxicological study was then carried out on 2:4-dichlorobenzyl alcohol as it appeared to be the least toxic of the dichloro-compounds.

TABLE III

LETHAL ACTIVITY OF SELECTED COMPOUNDS

Compound	Concentration (1 in −)	*Lethal time
Benzyl alcohol	50	1 hour
4-Chlorobenzyl alcohol	400	24 hours
2:4-Dichlorobenzyl alcohol	1000	1 hour
3:4-Dichlorobenzyl alcohol	1250	1 hour
3:4:5-Trichlorobenzyl alcohol	6000	24 hours
4-Bromobenzyl alcohol	450	24 hours
4-Methylbenzyl alcohol	200	24 hours
4-Chloro-3:5-dimethylbenzyl alcohol	1800	1 hour

* Time to reduce viable count to 100 per ml.

TABLE IV

APPROXIMATE LETHAL DOSES (LD 50) OF SELECTED COMPOUNDS TO MICE

Compound	Vehicle	Acute oral toxicity (mg./kg.)	Acute subcutaneous toxicity (mg./kg.)
Benzyl alcohol	Aqueous solution	1150	950
2:4-Dichlorobenzyl alcohol	Acacia suspension	1300	1770
3:4-Dichlorobenzyl alcohol	Acacia suspension	620	700

Short-term chronic toxicity. Three groups of newly-weaned albino rats, with five males and five females in each, were given daily oral doses in propylene glycol of 50, 150 or 500 mg./kg., 6 days each week for 3 weeks. An additional group was given propylene glycol alone as a control. The rats were weighed daily and those receiving the largest dose were examined for haematological effects during the last week of dosing. At autopsy the livers and kidneys were weighed and specimens of the major organs examined histologically.

Increases in weight were the same in all groups, and there were no pathological effects on blood or organs.

Local toxicity to rabbit eye. Two drops of a 0·08 per cent aqueous solution were instilled into the cupped eyelid of one eye of each rabbit, and maintained there for 60 seconds before the surplus solution was allowed to drain away; the other eye of each rabbit was treated with water as a control. Applications were made on 4 successive days to three rabbits without having any irritant effects.

Single applications of 1 and 5 per cent solutions in polyethylene glycol 400 and of a 1·5 per cent solution in propylene glycol had some irritant effects which were no greater than those produced by the solvents alone.

Skin sensitisation. Two methods were used to test for skin sensitisation in the guinea pig. In the first method, ten applications of a 2 per cent

solution in acetone were made to the shaved skin of three guinea pigs over a period of 3 weeks. The first dose was 0·05 ml. and the nine subsequent doses were 0·1 ml. each. Two weeks after the last sensitising dose the challenging dose of 0·05 ml. was given and the skin examined 24 hours later. There were no reactions from either the first sensitising or the challenging dose.

In the second method the dosage regime was similar except that a 0·1 per cent aqueous solution was given intradermally. The reaction produced in the sensitised guinea pigs was slight and no more than that produced by the first sensitising dose.

From this study it appears that 2:4-dichlorobenzyl alcohol has a low toxicity and shows good activity against a wide range of bacteria and moulds.

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DISCUSSION

The Paper was presented by MR. A. H. FENTON.

THE CHAIRMAN. Was any information available about the absorption of 2:4-dichlorobenzyl alcohol by rubber caps?

DR. K. R. CAPPER (London). The reference to the partition of 2:4-dichlorobenzyl alcohol in rubber in MR. SYKE's Paper indicated that the concentration might fall below the minimum inhibitory figure for Gram-positive bacteria. Do any other compounds show greater promise?

DR. F. HARTLEY (London). Was any information available about other properties of the compounds and of the stability of 2:4-dichlorobenzyl alcohol? If the halogen group was easily removed, incompatibilities might arise. In the case of emulsions it was important to know what chemical changes occurred if a substance was transferred from one phase to another.

DISCUSSION

PROFESSOR G. BROWNLEE (London). In Table IV the acute oral toxicity of an aqueous solution of benzyl alcohol was compared with that of an acacia suspension and until the range of the approximate lethal doses, was known it was difficult to say whether there was any difference between the first two compounds. Benzyl alcohol was more toxic in the subcutaneous tests, suggesting that it was poorly absorbed from the gastrointestinal tract. What was the absorption from under the skin? It would be useful to have the figures for the intravenous toxicity.

MR. G. SYKES (Nottingham). Absorption into rubber was not connected with chemical activity.

DR. J. C. PARKINSON (Brighton). Was 2:4-dichlorobenzyl alcohol stable when autoclaved?

MR. H. G. ROLFE (London). For what purpose was the substance intended to be used, as an ingredient of throat lozenges or of injections?

DR. H. S. BEAN (London). How active were the compounds in fairly high dilution? In addition to the lethal concentration it was necessary to know the concentration exponent.

DR. A. H. BECKETT (London). It seemed from the results that Ferguson's Law was applying as the activity was proportional to the degree of saturation in the biophase. Had any work been done on these compounds in the presence of non-ionic compounds which would easily inactivate phenols?

DR. A. M. COOK (London). Why had mixtures of different strains and species been used? As 2:4-dichlorobenzyl alcohol might be used in throat lozenges was there any further information on the spectrum of this compound particularly on diphtheroids and Gram-negative cocci? An organism resistant to a general bactericide might not be resistant to a chlorinated compound since there was a great deal of specificity with increasing chlorination.

DR. LESSEL replied. The number of animals and dose levels were insufficient to show whether there was a statistically significant difference between the acute subcutaneous and acute oral figures. It was difficult to compare results of aqueous solutions and of acacia suspensions. He agreed that the figures suggested that there was not a vast difference between benzyl alcohol and the 2:4-derivative. Work was in progress to establish the reason for the lower subcutaneous toxicities. Intravenous injections had not been used.

MR. FENTON replied. 2:4-Dichlorobenzyl alcohol was readily absorbed by rubber; the other compounds were not investigated in this connection. The compound would pass into the oil phase of an emulsion. In the neutral range it appeared to be stable in water. A 0·1 per cent aqueous solution at room temperature or at 37° for two months showed little change. He had no specific information about the stability of an aqueous solution on autoclaving. The material had been formulated in a throat lozenge but it might have application as a preservative or as a therapeutic

agent. It seemed that both anionic and non-ionic surface-active agents inactivated these compounds to some extent.

MR. CARTER replied. Mixtures of organisms had been used as a matter of expediency. The compound had been effective against a number of organisms common in the mouth and throat but it had not been tested against Gram-negative cocci, which do not cause infection in the throat.

THE INTERACTION OF CHELATING AGENTS WITH BACTERIA

PART I. 8-HYDROXYQUINOLINE (OXINE) AND *Staphylococcus aureus*

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The interaction of solutions containing oxine or iron or both (in a 1 : 1 molar ratio) with *Staph. aureus* is examined with respect to the chelating agent and metal ion binding by the bacteria. Iron enhances the amount of oxine uptake by the organism although the presence of the chelating agent does not affect the amount of iron bound. Iron is shown to be present in the ferric state in dilute solutions containing oxine. Two types of iron receptor sites in the bacterial surface are postulated and iron is shown to mediate oxine binding by the bacteria.

THE antibacterial activity of certain compounds for example 8-hydroxyquinoline, the tetracycline antibiotics, and *iso*nicotinic acid hydrazide, has been attributed at least in part, to their ability to chelate trace metals[1]. The importance of the latter in enzyme systems, and particularly those involving the metalloflavoproteins, is being increasingly realised.

Because various hypotheses have been advanced for the mechanism of antibacterial action of chelating agents, it is of interest to provide further information concerning the interaction of such substances with bacteria in the presence and absence of trace metals.

EXPERIMENTAL

Oxine. Analar 8-hydroxyquinoline (oxine) was recrystallised from ethanol, m.p. 76° (uncorr.), Heilbron and Bunbury[2] gave 75 to 76°; log ϵ 4·50 at λ max 240 mμ in water. *Ferrous ammonium sulphate.* Solutions of the Analar reagent in water were prepared immediately before use. *Metal depletion of media.* Distilled water, obtained from an all-glass still, was used throughout this work. Normal saline: 9 g. sodium chloride (Analar) was dissolved in 250 ml. of water and 1 ml. chloroform containing 25 μg. of oxine was added. The solution was shaken vigorously and set aside one hour before extraction with chloroform (3 × 25 ml. portions). This treatment with oxine was repeated thrice. The solution was finally extracted with 6 × 25 ml. portions of chloroform to remove all traces of oxine, boiled to expel dissolved solvent, cooled and diluted to 1000 ml. with water. *Spectrophotometer.* A Hilger H 700 spectrophotometer was used in conjunction with matched fused silica cuvettes. *Organism. Staphylococcus aureus* (originally N.C.T.C. 6571) was maintained on nutrient agar slopes. Cultures were incubated for 18 to 24 hours at 37°.

Preparation of bacterial suspensions. The bacteria were harvested from slope cultures using metal-depleted normal saline; the suspension was centrifuged at 8500 *g* for 10 minutes and the cells resuspended and again washed with the same medium. The bacteria were finally suspended in

metal-depleted normal saline at a concentration of 165×10^8 organisms/ml. Routine standardisation was carried out nephelometrically.

Preparation of suspensions of heat-killed bacteria. Washed suspensions of *Staph. aureus* (approx. 165×10^8 organisms/ml.) in metal-depleted normal saline were maintained at 70° for 30 minutes. The bacteria were centrifuged, washed twice and resuspended in metal-depleted saline (165×10^8 organisms/ml.).

Preparation of suspensions of isolated cell walls. Suspensions of isolated cell walls of *Staph. aureus* were prepared following the method of Salton and Horne[3].

Preparation of iron-oxine solutions. Solutions were prepared to contain 1 μg./ml. oxine and 2·7 μg./ml. ferrous ammonium sulphate, that is, oxine and iron in a 1 : 1 molar ratio. Spectrophotometric examination of the solution (4 cm. cuvettes) revealed the presence of a single absorption peak at 241 mμ which underwent a gradual bathochromic shift to 248 mμ on standing. The intensity of the latter peak reached a steady figure after 48 hours; all oxine-iron solutions were, therefore, stored for 48 hours before use.

General Method for Drug-bacteria Contact

Solutions containing oxine or iron, or both, were introduced into glass centrifuge tubes which were immersed in a water bath maintained at 25° (±1°). The total volume was adjusted to 42 ml. with water. 3 ml. volumes of bacterial suspension were added to each solution after allowing sufficient time for temperature equilibration. The final concentration of bacteria in the test suspension was 11×10^6 organisms/ml. unless otherwise stated. After 60 minutes, unless otherwise specified, the bacteria were removed from the suspension by centrifuging twice at 8500 *g* for 10 minutes before spectrophotometric examination of the supernatant solution between 220 to 300 mμ (1 cm. cuvettes).

Determination of Unchanged Oxine in Solutions after Contact with Bacteria

The intensity of the ultra-violet absorption spectra of the reaction solutions was frequently greater than that attributable to the initial concentration of oxine. This was due to the liberation from the bacteria of cellular constituents having λ max 260 mμ; these substances will be referred to as cell exudate[4]. Solutions containing cell exudate are unaffected by extraction of the solutions with chloroform, whereas oxine is completely removed from an aqueous solution by this means. This separation may be effected quantitatively and the oxine concentration determined by subtraction of the ultra-violet absorption spectrum of the chloroform-extracted solution from that of the initial reaction solution. 20 ml. portions of the reaction solutions were extracted with chloroform (reagent grade, 5 × 20 ml.) and a total volume of 20 ml. of water was used for washing. The solvent was removed from the aqueous layer by boiling and the final volume was re-adjusted to 20·0 ml. after cooling.

Colorimetric Determination of Iron

Iron was determined by formation of the 3 : 1 ferrous-*o*-phenanthroline complex (log $K_s 21{\cdot}3$[5]) in the presence of a reducing agent.

The concentration of iron remaining in solution after contact with suspensions of *Staph. aureus* was relatively low but the following procedure was found to give satisfactory results under these conditions. To a suitable volume of the solution was added 4·0 ml. of a 4 per cent w/v solution of hydroxylamine hydrochloride in water followed by 2·0 ml. of a 0·4 per cent w/v solution of *o*-phenanthroline in 50 per cent v/v aqueous methanol. The final volume was adjusted to 100 ml. and the optical density of the solution measured at λ max 510 mμ in 4 cm. cuvettes. Colour development was complete within a few minutes and the optical density at 510 mμ was unchanged after 24 hours at room temperature.

This method also proved satisfactory for the determination of iron *in the presence of oxine*, using an appropriate calibration curve.

Recovery of Iron from Bacterial Suspensions

Staph. aureus suspensions were exposed to solutions of ferrous ammonium sulphate (18·66 μg./ml.) for known periods of time (between 2 to 60 minutes); solutions of hydroxylamine hydrochloride and *o*-phenanthroline were then added, the final concentrations of these reagents being identical with those used for the colorimetric determination of iron. After a further 15 minutes, the bacteria were removed by centrifuging and the optical density of the supernatant solution measured at 510 mμ.

Reaction of Staph. aureus *with Solutions containing Iron-phenanthroline Complex*

The bacteria were added to solutions containing iron (2·5 to 20 μg./ml.), hydroxylamine hydrochloride and *o*-phenanthroline in the same concentrations as those used for the colorimetric determination of iron. After 15 minutes the bacteria were removed by centrifuging and the supernatant solutions were examined spectrophotometrically at 510 mμ.

Results

The Uptake of Oxine by Staph. aureus

Rate of uptake. The amount of oxine bound by *Staph. aureus* from a solution containing 14·9 μg./ml. was independent of the contact time between 10 and 60 minutes. The supernatant solution contained 14·05 μg./ml.

Relationship between the uptake of oxine and its initial concentration in solution. Figure 1 shows results obtained using solutions initially containing up to 15 μg./ml. of oxine. More concentrated drug solutions were not examined because the accuracy of the results obtained would have been adversely affected. At an initial concentration of 9·33 μg./ml., 6·3 per cent of the available oxine was bound by the bacteria. The amount of oxine bound by isolated cell walls from *Staph. aureus* is also shown in Figure 1.

The Uptake of Iron by Staph. aureus

The results obtained for ferrous ammonium sulphate solutions (up to 20 μg./ml. initially)* and whole cell and isolated cell wall preparations are shown in Table I.

A very slight increase in the amount of iron bound by whole cells (maximum 3·5 per cent) from solutions initially containing 12 to 19 μg./ml. ferrous iron was observed in the presence of 1 μg./ml. of oxine. These solutions were stored before use.

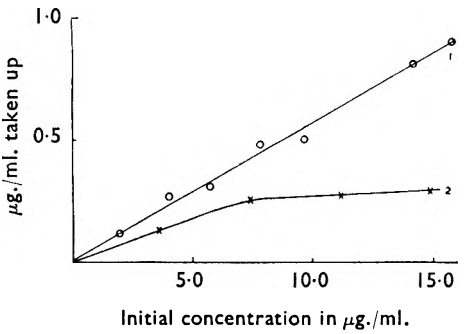


FIG. 1. Uptake of oxine by whole cells (curve 1) and isolated cell walls (curve 2) of *Staph. aureus*.

The results using a solution of ferrous ammonium sulphate (18·66 μg./ml.) and contact times of between 2 to 60 minutes (see Figure 2) indicate a rapid initial uptake followed by a relatively slow uptake of iron. No saturation point was reached within 60 minutes contact time.

TABLE I

COMPARISON OF THE AMOUNT OF IRON BOUND BY WHOLE CELLS AND ISOLATED CELL WALLS OF *Staph. aureus*

μg./ml. available	μg./ml. taken up by			Percentage uptake (compared with whole, viable cells) by	
	Whole viable cells	Heat-killed cells	Isolated cell walls	Heat-killed cells	Isolated cell walls
4·66	4·49	3·39	2·58	75·5	57·6
9·33	8·80	6·41	4·33	73·0	49·2
14·0	12·66	9·20	5·60	72·6	44·3
18·66	16·41	11·83	6·39	72·1	39·0

Recovery of Iron from Staph. aureus *Suspensions*

The concentrations recoverable after 2 to 60 minutes contact time, under conditions comparable to those used in the previous section (see Fig. 2) decreased with increasing time of contact and also decreased at a much faster rate than that at which iron was taken up by the organisms. Table II presents the results for a fixed contact time (60 minutes) and ferrous ammonium sulphate solutions initially containing 2 to 19 μg./ml.

The Reaction of Staph. aureus *with Solutions containing Iron and Oxine (1 : 1 Molar Ratio)*

After removal of the bacteria from the contact suspension (λ max of the initial solution 248 mμ), the supernatant solution (curve 2 of Fig. 3) contained a component having an absorption spectrum (λ max 240 mμ) resembling that of oxine. This solution, after addition of iron and 24

* The iron concentrations are expressed as ferrous ammonium sulphate throughout this paper.

hours storage again showed an absorption maximum at 248 mμ. The amount of oxine bound (0·933 μg./ml. available) in the presence of iron was 0·373 μg./ml. or 40 per cent of that available. The time taken for the bacteria to effect the hypsochromic shift of the iron-oxine solution (248 to 240 mμ) was 15 minutes; the absorption peak shifted to 240 to 241 mμ within 5 minutes.

TABLE II

IRON BINDING BY *Staph. aureus* IN THE ABSENCE OF OXINE

Iron available μg./ml.	Iron recovered μg./ml. (*a*)	Iron* bound as complex μg./ml. (*b*)	Sum of (*a*) and (*b*)	Iron non-recoverable μg./ml.
2·33	0·8	0·33	1·13	1·20
4·66	1·4	0·66	2·06	2·60
7·0	2·15	0·96	3·11	3·89
9·33	3·2	1·33	4·53	4·80
11·66	5·23	1·55	6·78	4·88
14·0	6·78	1·90	8·68	5·32
16·33	8·28	2·23	10·51	5·82
18·66	10·0	2·46	12·46	6·20

* Ferrous-*o*-phenanthroline complex.

Reaction of Staph. aureus *with Solutions containing Iron and* o-*Phenanthroline*

The results are presented in Table II. This Table shows the corresponding amounts of iron which could be recovered with *o*-phenanthroline at the same initial concentration levels of iron.

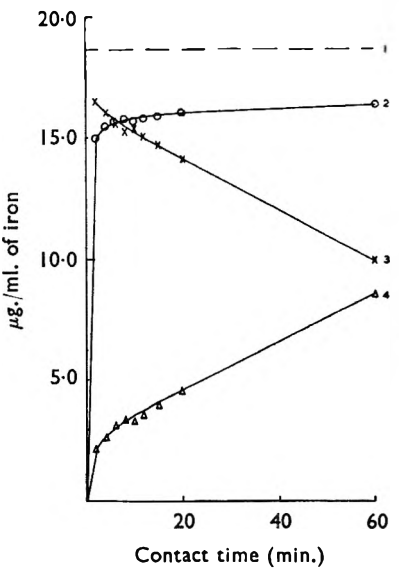


FIG. 2. Interaction of iron with *Staph. aureus*.
Curve 1, the amount of iron available.
Curve 2, the uptake of iron.
Curve 3, the recovery of iron using *o*-phenanthroline.
Curve 4, the amount of iron non-recoverable from the bacteria.

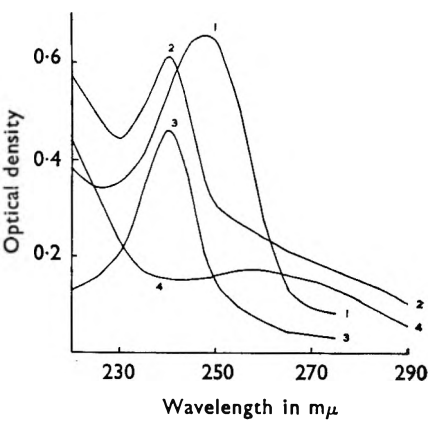


FIG. 3. Ultra-violet absorption curves of solutions containing iron and oxine (1:1 molar ratio) before and after contact with *Staph. aureus*.
Curve 1, reference solution.
Curve 2, the supernatant solution after removal of the bacteria from the contact suspension.
Curve 3, curve 2 corrected for the presence of cell exudate (curve 4).

Experiments with Heat-killed Bacteria

Reaction with iron-oxine solution. The absorption spectrum of the supernatant solution, corrected for the presence of exudate, showed a peak at 248 mμ. The amount of oxine bound accounted for 54 per cent of the initial concentration.

Rate of uptake of iron. In contrast with the results obtained with viable organisms, iron uptake by heat-killed cells was complete after 3 minutes contact time.

Uptake of iron in presence and absence of oxine. There was very little difference in the amount of iron bound by heat-killed cells in the presence and absence of oxine, as was also the case using viable cells. However, more iron was bound by living organisms, as may be seen in Table II.

Recovery of iron with o-phenanthroline. After 2 minutes contact time, 9·9 per cent of the iron available (18·66 μg./ml.) could not be recovered from the bacteria with *o*-phenanthroline. This figure increased to only 11·6 per cent after 60 minutes contact with heat-killed bacteria.

Discussion

Analytical Methods

In related unpublished studies with several antibacterial drugs and cell exudate from different bacteria, it has been shown that the method used in the present work effects the quantitative separation of the drug from the exudate. The method has the advantage that the spectrophotometric identity of the components may be verified.

State of Oxidation of Iron in Iron-Oxine Solutions

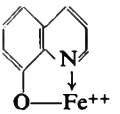
The iron-oxine solutions were prepared from fresh solutions of ferrous ammonium sulphate in water. The ultra-violet absorption spectrum of the iron-oxine solution (1 : 1 molar ratio) underwent a gradual bathochromic shift on standing which could be attributed to (*a*) slow chelation, (*b*) slow oxidation of the ferrous iron and subsequent chelation with oxine or (*c*) formation of a ferrous-oxine chelate and subsequent oxidation of the metal ion. The location of the absorption peak observed on addition of iron, in the ferric state, to a solution of oxine was 248 mμ; thus, *the iron-oxine solutions examined contained ferric iron.* Colorimetric checks using *o*-phenanthroline in the absence of a reducing agent demonstrated the disappearance of ferrous iron in the presence of oxine. (A ferrous salt was used to prepare the solutions because it is easier to handle in neutral aqueous solutions than a ferric salt.)

The valency state of the iron offered to the bacteria appears to be unimportant, as the extent of iron binding was identical whether ferrous (ferrous ammonium sulphate) or ferric (ferrous ammonium sulphate and oxine) iron solutions were used. Further, the actual valency state of the iron bound at the bacterial surface is not known and cannot be inferred from a knowledge of the valency state of iron in the bulk phase.

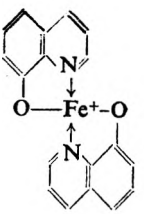
Ferric Iron-Oxine Complexes

The relative proportion of the complex(es) present in the iron-oxine solutions (1 : 1 molar ratio) used in the present work is not yet clarified.

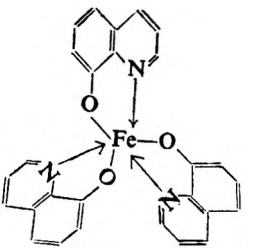
Three are possible, namely, the charged 1:1 (I) and 1:2 (II) and the electrochemically neutral 1:3 (III) complexes.



(I)



(II)



(III)

Oxine Binding by Staph. aureus

Relatively little oxine is bound by the bacteria in the absence of added heavy metal ions. Possibly, the binding observed is due to residual traces of heavy metals in the system. The uptake of the chelating agent from solution is rapid and is complete within 10 minutes of addition of the bacteria. Secondly (see Fig. 1), less than half the amount of oxine bound by whole cells is taken up by isolated cell walls. Standardisation of cell wall preparations, in terms of the number of organisms from which they were derived, is difficult and losses inevitably occur during isolation. Further, almost double the surface area may be made available for binding on separation from the intact bacteria. Therefore, the percentage of oxine bound by isolated cell walls may bear little relation to the amount bound by the same structure in viable bacteria. Probably, the drug is bound preferentially by the cytoplasmic membrane, at least in the living organism.

Iron Binding by Staph. aureus

The relationship between the uptake of iron and the time of contact with *Staph. aureus* indicates at least two mechanisms of binding. Most of the iron was bound within 10 minutes of contact with the bacteria but no saturation was observed after a further 50 minutes. The sum of the amount of iron recovered from the cells and the amount of iron bound as the ferrous-*o*-phenanthroline complex (see Table II) does not correspond with the total iron bound by *Staph. aureus*. Possibly iron gradually penetrates the bacterial cell, or alternatively, some molecular rearrangement occurs at the surface of the cell which results in an increase in the iron binding forces. It is significant that oxine does not affect the final extent of iron binding (see later under mechanism of antibacterial action).

The percentages of iron bound by isolated cell walls of *Staph. aureus* in relation to those observed for the intact organisms (cf. Table II) are subject to the same comments as those made in the case of oxine.

Oxine Binding in the Presence of Iron

The ultra-violet absorption curves presented in Figure 3, indicate that the bacteria remove iron from its complex with oxine in aqueous solution. This reaction was rapid, being completed within 15 minutes. Thus some component of the bacterial surface (cytoplasmic membrane) was capable of binding iron *more firmly* than oxine under the conditions of these experiments. *However, the presence of iron considerably increased the amount of oxine bound by* Staph. aureus from 6 to 40 per cent of the total available oxine; in the presence of a ten molar excess of iron, the increase was only to 15 per cent of the total available oxine.

When iron was presented to *Staph. aureus* as the ferrous-*o*-phenanthroline complex, the bacteria did not bind the metal preferentially. Either the stability constant of the complex is too great or the bacteria actually bind iron in the ferric state only; alternatively steric factors may prevent any interaction of the complexed metal ion with the receptor sites of the bacterial surface.

Results Using Heat-killed Bacteria

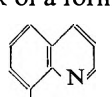
Heat-killed bacteria appear to be incapable of removing iron from its complex with oxine and in fact the complex itself is bound by the cells. The amount of iron bound by heat-killed cells is also reduced compared with viable cells and uptake is complete within 10 minutes. Further, the amount of iron recoverable from the cells with *o*-phenanthroline was virtually the same after initial contact times of 2 to 60 minutes. Only about 10 per cent of the iron available to the bacteria could not be recovered by this method.

General Discussion

Two types of iron binding sites are postulated at the cytoplasmic membrane of *Staph. aureus* to account for the results described, the first capable of loosely binding iron by ionic forces ("anionic receptor sites") and the second capable of firmer binding, possibly involving chelation ("iron chelating sites"). Similar sites may also be present in isolated cell walls but as the site of toxic action of oxine-metal complexes is probably the cytoplasmic membrane, the discussion will be restricted to this structure.

The "anionic receptor site" is capable of binding hydrated positively charged ferrous ions and charged ferric iron-oxine complexes (I and II), because the amount of iron bound remains unchanged in the presence of oxine and yet the oxine uptake increases in the presence of iron.

The limited extent of oxine binding in the absence of added heavy metal ions may be due to either the lack of a formal charge on the oxine molecule



(IV)

(cf. the difference between IV and I) reducing the possibility of binding to anionic (or cationic) sites in the cell surface, or alternatively to the lack

of a heavy metal ion to mediate binding. If the latter were true, no oxine would be bound in the complete absence of heavy metal ions but there is no known method for removing every trace of such metals from a medium.

As the charged iron-oxine complex is capable of binding to the "anionic receptor site," it is reasonable to assume iron mediation of oxine binding by formation of a ternary oxine-metal-receptor complex. Such a concept accounts for the observed increase in oxine binding on addition of metal ions. The work of Klotz and his associates[6] on the facilitated binding of dye molecules to proteins in the presence of metal ions may be cited in support of this hypothesis.

The decreased uptake of oxine on addition of increasing molar proportions of iron indicates competition between the hydrated metal ions and the charged metal-oxine complexes (I and II) for the "anionic receptor sites." Possibly, van der Waals' forces between the aromatic nucleus and complementary receptor surface may reinforce the ionic attractive forces, since not all the oxine is displaced by a large molar excess of iron.

The presence of a second type of iron binding receptor, probably of chelating character, is postulated for three reasons.

Firstly, the bacteria can compete successfully for oxine chelated iron, stability constants log K_1 12·3, log K_2 11·3 and log K_3 10·3[7], under the conditions employed.

The alternative explanation, that free iron penetrates the surface, thus being removed from the equilibrium with oxine, is precluded since only slow penetration of the bacterial surface occurs with free iron and bacteria rapidly remove iron from its complex with oxine. Probably the iron-oxine complex is attracted to the surface where iron is bound at the "iron chelating sites" and oxine liberated into solution.

Secondly, even after short periods of iron-bacteria contact, a proportion of the iron cannot be recovered from the bacteria with *o*-phenanthroline (log K_3 21·3[5]).

Thirdly, heat-killed bacteria bind less iron than viable cells and nearly all may be recovered with *o*-phenanthroline. These bacteria are no longer capable of removing iron from its complex with oxine, probably due to modification of the "iron chelating sites." If these "iron chelating sites" are anionic in character, heating apparently only destroys their chelating ability, since binding of 5-aminoacridine, as a cation, to the same organism is unaffected by similar treatment[8].

That chelation of trace heavy metals, iron and copper, is implicated in the antibacterial action of oxine was demonstrated when the seven other position isomers of oxine were found to be inactive and oxine itself was devoid of activity in heavy metal depleted media[9]. Since the actual antibacterial activity of iron and oxine apparently varies with their molar ratios, it has been suggested that an equilibrium exists between the lipophilic 1:2 (*ferrous*-oxine) and the hydrophilic 1:1 (*ferrous*-oxine) complexes and that, whereas the former facilitates membrane penetration, the latter is the toxic entity. Recently[1], the charged 1:1 (I) and 1:2 (II) and the electrochemically neutral 1:3 (III) *ferric* iron-oxine complexes

have been considered in this respect. The ability to form a lipophilic complex is considered a necessary property if an oxine derivative is to show significant antibacterial activity (cf. work with azaoxines[7,10,11]); an intracellular site of action was postulated.

If a 1:3 iron-oxine complex, because of its lipid solubility, penetrated the bacterial cell from a 1:1 molar iron-oxine solution, one would expect liberation of *free iron* into the biophase unless the iron were also held at a site in the cell wall or cytoplasmic membrane. The fact that *free oxine* is speedily liberated into the biophase upon presentation of the above solution to the bacteria indicates that at least a gross migration of the 1:3 complex across a lipoid boundary has not occurred. The possibility of the chelate acting at the cytoplasmic membrane must, therefore, be considered. Addition of iron does not reduce the bactericidal activity of an iron-oxine solution (1:1 molar ratio) under conditions identical to those described for the uptake measurements (unpublished work). Because we have shown that oxine is not implicated in the uptake of iron, whereas iron is involved in the uptake of oxine, it seems possible, contrary to current views, that the antibacterial activity of oxine in the presence but not the absence of iron is due to the latter constituting a bridge to bind the drug to an important site in the cytoplasmic membrane. The iron-oxine complex may be regarded as interacting in an enzyme system in a manner analogous to the metal of a metalloflavoprotein and its substrate reacting with the rest of the flavoprotein; such an interaction could inhibit the normal processes of electron transfer with consequent impairment of enzyme function. The oxine inhibitor derived from erythrocytes (erythrochelatin[12,13]) probably protects bacteria against this inhibition of enzyme function since it has been demonstrated not to interfere with iron-oxine chelation.

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DISCUSSION

The Paper was presented by DR. A. E. ROBINSON.

THE CHAIRMAN. Were the bacteriostatic or bactericidal concentrations of oxine diminished in the presence of iron? What was the appropriate

calibration curve used for the determination of iron in the presence of oxine and how was it prepared? In Fig. 2 what does 'available' iron mean?

MR. G. SYKES (Nottingham). Excessive concentrations of certain metals may turn the metallic compound from a nutrient to an antangonist. Was an extra oxidation-reduction system being introduced? It seemed unnecessary at present to consider the idea of "anionic receptor sites" and "iron chelating sites". He thought that the latter was possibly an enzyme or a unit of an enzyme. Care must be exercised in contrasting the activities of live and heat-killed organisms.

DR. L. SAUNDERS (London). Was the assumption that the exudate gave no chloroform-soluble extractive justified? Had the use of chelating ion exchange resins which could effectively remove traces of copper from solution been considered?

MR. H. D. C. RAPSON (Betchworth). Was the uptake of oxine fast or slow?

DR. A. M. COOK (London). Did the excess iron have any effect on the bactericidal activity of the oxine-iron complex? Had the use of *Strep. faecalis* been considered where the iron-containing enzymes were probably not so important as in the very aerobic *Staph. aureus*?

DR. ROBINSON replied. The use of bacteriostatic tests had been deliberately avoided since metal ions were present and the results would not be significant in relation to the uptake work. Preliminary results showed that iron had a very marked effect on the bactericidal activity of iron-oxine solutions. The appropriate curve for the determination of iron in the presence of oxine meant that allowance had been made for the slight deviation from the calibration curve for iron and *o*-phenanthroline observed in the presence of oxine. The "available" iron in Fig. 2 was the amount added initially. The "iron chelating sites" might form an integral part of an enzyme system, but the Authors preferred to use a general, rather than a specific, term at this stage. Structural changes undoubtedly occurred in the cytoplasmic membrane on heat-killing bacteria but work with 5-aminoacridine had indicated that an anionic centre survived the treatment. She was satisfied that cell exudate could be separated from the unchanged drug by the method used. Other methods for removal of trace metals had not been tried. The uptake of oxine was complete within 10 minutes. Bactericidal evaluation of the system was not yet complete but the addition of further iron to an iron-oxine solution could alter the death time of the bacteria in that solution from 130 to 45 minutes. Complete data was not yet available for any other organism.

THE DETERMINATION OF PHENOLIC COMPOUNDS IN PHARMACEUTICAL PREPARATIONS USING 4-AMINOPHENAZONE

By C. A. Johnson and R. A. Savidge

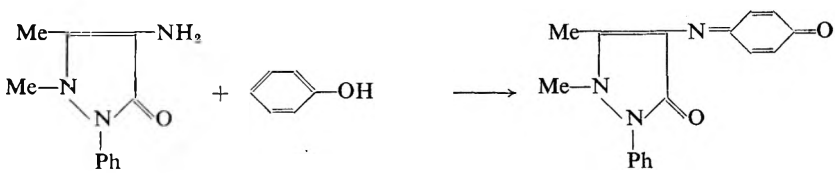
From the Analytical Development Group, Standards Department, Boots Pure Drug Co., Ltd.

Received May 30, 1958

The determination of phenols based upon coupling with 4-aminophenazone in the presence of an alkaline oxidising agent has been examined. The importance of pH has been studied and a suitable buffering agent has been recommended. The reactivity of many phenols of pharmaceutical interest has been investigated and the application of the method to a number of pharmaceutical preparations is described.

Until recently phenolic preservatives in injection solutions were determined exclusively by steam distillation of the phenolic material followed by bromination. An ultra-violet absorption method was frequently invalidated because the other ingredients of the injection solution themselves absorbed strongly at the critical wavelength, but a considerable advance was made in the application of this technique when the use of oxidised cellulose for the removal of certain interfering substances was introduced[1]. Many injection solutions still remain, however, to which this technique is inapplicable and a search was therefore made for a suitable method of determining phenolic substances which would be both accurate and rapid.

Such a method is based on the use of 4-aminophenazone[2]; the determination depends upon the production of a dye by the action of 4-aminophenazone on the phenol in the presence of an alkaline oxidising agent, usually potassium ferricyanide.



The method was first described by Emerson[2], discussed by Ettinger and others[3], and applied to the determination of phenols in waters from coke wastes by Shaw[4], and in industrial waste waters by Mohler and Jacobs[5]; much valuable information has been published by these workers. The present paper reviews the necessary conditions for quantitative reaction and in particular stresses the careful control of pH which is required; it also examines the applicability of the method to many phenols likely to be met in pharmaceutical practice and describes methods for the determination of many of these substances in typical pharmaceutical preparations.

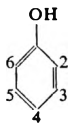
Scope and Applicability of the Method

In general substances having a free phenolic hydroxyl group and a free *para* position or a *para* position which is substituted by a halogen, hydroxyl or alkoxyl, sulphonic acid or carboxylic acid group give a positive reaction. Presumably these substituents are expelled in the reaction, for thymol and chlorothymol, which has the halogen in the *para* position to the hydroxyl, give coloured products which have identical absorption curves except that the molecular extinctions differ, consistent with the difference in molecular weights. A nitro or a carboxylic acid group in the *ortho* position relative to the free hydroxyl prevents the formation of colour and this is probably due to hydrogen bonding with consequent loss of phenolic character.

The reaction apparently occurs only at the *para* position, since there are several examples of substances having free *ortho* positions which give no colour, for example, methyl *p*-hydroxybenzoate, stilboestrol and *p*-cresol.

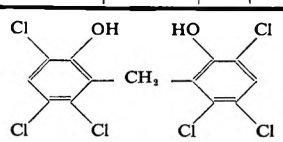
TABLE I

COLOURS DEVELOPED BY PHENOLIC COMPOUNDS OF PHARMACEUTICAL INTEREST



Substance	Substituents					Resulting colour in chloroform
	2	3	4	5	6	
Phenol						Strong orange colour
Chlorocresol		CH_3	Cl			Strong orange colour
o-Cresol	CH_3					Strong orange colour
m-Cresol		CH_3				Strong orange colour
p-Cresol			CH_3			No reaction
Chloroxylenol		CH_3	Cl	CH_3		Strong plum colour
Thymol	$CH(CH_3)_2$			CH_3		Moderately strong yellow colour
Chlorothymol	$CH(CH_3)_2$		Cl	CH_3		Moderately strong yellow colour
o-Amyl *m*-cresol	C_5H_{11}			CH_3		Strong orange colour
Salicylic acid	COOH					No reaction
Methyl salicylate	$COOCH_3$					Strong reddish-orange colour
Salol	$COOC_6H_5$					Strong reddish-orange colour
Salicylamide	$CONH_2$					Strong reddish-orange colour
p-Hydroxybenzoic acid			COOH			Strong orange colour
Methyl *p*-hydroxybenzoate			$COOCH_3$			No reaction
Propyl *p*-hydroxybenzoate			$COOC_3H_7$			No reaction
Resorcinol		OH				Brownish-orange colour
Hexylresorcinol	C_6H_{13}			OH		Brownish-orange colour
Guaiacol	OCH_3					Strong orange colour
Vanillin	OCH_3		CHO			No reaction
2:4:6-Trichlorophenol	Cl		Cl		Cl	Strong orange colour
Stilboestrol			X			No reaction
Hexoestrol			X			No reaction
Morphine			X	X	X	No reaction
Hexachlorophene*						Strong orange colours—
Dichlorophene†						insoluble in chloroform

* Hexachlorophene is



† Dichlorophene is

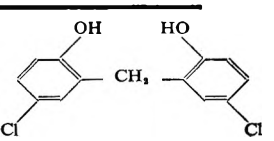


Table I shows the results obtained with a number of phenolic substances of pharmaceutical interest. In addition to the compounds mentioned, pyridoxine hydrochloride yields a strong plum colour which fades rapidly.

One or two of the substances listed call for special mention. As is to be expected, both *o* and *m*-cresol give strong colours while pure *p*-cresol does not react; this means that Cresol B.P., which is a mixture in which the *meta* isomer predominates, can only be determined in preparations with accuracy if a sample of the batch used in manufacture is available for use as a standard. The two dihydric phenols listed give rather unsatisfactory colours since the darkening characteristic of these substances in alkaline solution considerably modifies the orange colour. In general the colours produced are unstable in aqueous solution, some loss in intensity being observable after 15 minutes, but most of those deriving from pharmaceutical phenols may be readily extracted into chloroform, in which solvent they form stable solutions.

General Method

For Phenols giving Aminophenazone Dyes Soluble in Chloroform

Transfer a suitable aliquot of the prepared solution containing 0·2 to 0·4 mg. of the phenol to a 150 ml. separator. Add 1 ml. of 4-*aminophenazone solution* and wash in with sufficient *dilute ammonia buffer* to give a volume of approximately 50 ml. Add 1 ml. of *potassium ferricyanide solution* and mix. Extract the solution with 25 ml. of chloroform followed by two shakings each of 10 ml. passing each extract into a dry 50 ml. graduated flask through a small plug of cotton wool previously moistened with chloroform. Dilute to 50 ml. with chloroform, mix and read the optical density in a 1 cm. cell using an Ilford No. 602 filter and chloroform in the comparison cell. For phenol use a 0·5 cm. cell.

For Phenols giving Aminophenazone Dyes Insoluble in Chloroform

Transfer a suitable aliquot of the prepared solution containing 0·2 to 0·4 mg. of the phenol to a 50 ml. graduated flask and add 1 ml. of 4-*aminophenazone solution*. Wash in with *dilute ammonia buffer* to produce a volume of about 45 ml. Mix and add 1 ml. of *potassium ferricyanide solution* and dilute to 50 ml. with the dilute buffer. Mix and read the optical density as rapidly as possible in a 1 cm. cell using an Ilford No. 603 filter and a suitable aliquot of the prepared solution, diluted with dilute ammonia buffer to 50 ml. in the comparison cell.

Reagents. 4-*Aminophenazone solution.* Dissolve 0·5 g. in 25 ml. of water, shake and filter. This solution is stable for 2 to 3 days. *Potassium ferricyanide solution.* Dissolve 2 g. of potassium ferricyanide in 25 ml. of water. This solution should be prepared daily. *Strong ammonia buffer.* Dissolve 67·5 g. of ammonium chloride in 570 ml. of strong solution of ammonia and dilute to 1 litre with water. *Dilute ammonia buffer.* Dilute 2 ml. of strong ammonia buffer to 1 litre with water.

Some Observations on the General Method

The method, as described above, has been based on considerable experimental work to determine the optimum conditions, particularly of pH and of quantity of ferricyanide solution added.

The effect of pH was examined by carrying out the method on a solution of chlorocresol at each of 9 pH levels between 4 and 12. Simultaneously control determinations were carried out using no chlorocresol. As can be seen from Figure 1, the blank value is very high in aqueous and quite appreciable in chloroformic solutions at pH values below 9 and at values greater than 10 the maximum colour is not obtained. In aqueous solutions the very high blank at pH values below 9 is due to the formation of "antipyrine red". Earlier workers[3,6,7] have suggested various means of adjusting the reaction solution to a pH of about 10 but unless the solution is buffered the pH may fall to a value as low as 7 or 8 on reaction, which may lead to erroneous results. For this reason an ammonia buffer solution has been recommended and in the many determinations which have been carried out by this procedure the initial pH has been about

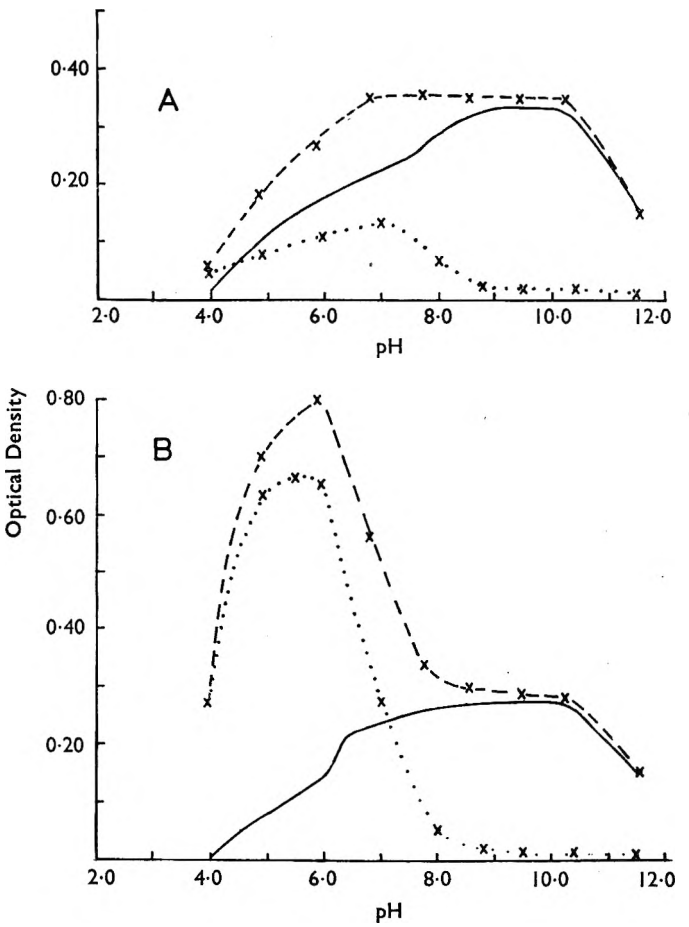


FIG. 1. The effect of pH on the colour formed with chlorocresol. A, in chloroform; B, in water; – – – – – reagents and chlorocresol; . . . . . reagents only; ——— difference due to chlorocresol.

10 and has never fallen below 9·4; this means that the pH is maintained safely within the permitted limits (Fig. 1).

The quantity of potassium ferricyanide required has also been studied in detail. The recommended amount ensures that sufficient is present without excess. The excess has little effect on the chloroform extraction procedure but it contributes to an increased blank value in the aqueous method. The choice of order of addition of reagents recommended by Ettinger[3] has been confirmed; it is important to prevent the aminophenazone reagent and the ferricyanide from coming into contact with each other except in the buffered solution. Temperature changes within the usual laboratory limits have no significant effect on the intensity of colour produced. In aqueous solutions the colour of the blank slowly increases whilst that of the sample slowly fades and the extinction value of the solution should be measured within 5 minutes if this method is being used; in chloroform the colours are stable over long periods and little or no change has been observed after 24 hours.

The absorption characteristics of each substance considered have also been examined and curves from 400 to 600 mμ have been prepared in both aqueous and, where possible, chloroformic solutions. In aqueous solutions most phenols show a broad peak in the region of 480 to 500 mμ but in chloroform there is a fairly definite maximum. This occurs at about 450 mμ for phenol itself and for most of the other substances listed in Table I giving an orange colour. With salicylamide and methyl salicylate the peak occurs at about 490 mμ and for chloroxylenol at about 510 mμ. Beer's Law is obeyed over the range 0 to 0·4 mg. for all those substances listed giving colours (with the exception of the two dihydric phenols).

Application of the Method to Pharmaceutical Preparations

The general method has been applied to the determination of the phenolic constituents in injection solutions and a considerable number of other pharmaceutical preparations. A selection from these is given below.

Injection Solutions

The phenolic preservative in many injection solutions has been determined by a direct application of the method described above. The following general procedure is applicable:

Dilute 1 ml. of injection solution to 100 ml. with *dilute ammonia buffer*, mix thoroughly, filter if necessary, and transfer a suitable aliquot to contain about 0·2 to 0·4 mg. of the phenol to a 150 ml. separator and continue as described in the general method (chloroform extraction procedure) beginning with the words "Add 1 ml. of *4-aminophenazone solution*. . . ".

Some typical recovery results are shown in Table II. In addition to the examples listed a sample of Injection of Ascorbic Acid was prepared in the laboratory to contain 5 mg. of phenol per ml. Ten determinations were made on this sample (five on each of two successive days) and a mean recovery of 101·1 per cent of the expected value was obtained (standard deviation 0·467).

One series of injection solutions, the Insulin Zinc Suspensions, contain methyl *p*-hydroxybenzoate (methylparaben) as a preservative and this does not couple with aminophenazone. In this case a preliminary hydrolysis is necessary similar to that described below for the determination of methylparaben in a suspension for oral use.

TABLE II

RECOVERY OF PHENOLIC BACTERIOSTATS FROM INJECTION SOLUTIONS

Injection solution	Content of Bacteriostat (mg. per millilitre)					
	Nominal		Found	Added	Found	Recovery per cent
Aneurine hydrochloride ..	Cresol	3 mg.	3·20	2·0	5·22	101
Bismuth	Chlorocresol	1 mg.	1·08	2·0	3·11	101·5
Bismuth oxychloride* ..	Chlorocresol	1 mg.	0·60	1·0	1·62	101
Cyanocobalamin	Phenol	5 mg.	5·20	2·5	7·80	104
Concentrated liver* ..	Chlorocresol	1 mg.	0·80	1·0	1·78	98
Heparin*	Cresol	3 mg.	2·40	2·0	4·44	102
Insulin	Phenol	2·5 mg.	2·60	2·0	4·58	99
Insulin zinc suspension ..	Methyl *p*-hydroxybenzoate	1 mg.	0·90	2·0	2·84	97
Stilboestrol dipropionate ..	Phenol	6 mg.	6·15	2·0	8·20	102·5

* These samples were several months old.

Injection of Stilboestrol Dipropionate B. Vet. C. is an example of an oily injection and in such a case the bacteriostat may be determined by the method described below for Carbolised Oil B.P.C., 1 g. of sample and a 50 ml. aliquot of the solution after dilution to 1 litre is satisfactory.

Assuming, as is usually the case, the direct application method can be used, the whole determination can be carried out within 15 or 20 minutes and this compares very favourably with the time required for a steam distillation and bromination assay. Providing attention is paid to certain details such as the adequate washing down of the reagents, the method is capable of yielding accurate results in the hands of junior personnel. In the extensive series of recovery experiments which have been made it seems evident that the method is capable of yielding results well within $\pm$ 3 per cent of the true figure.

Solution of Chloroxylenol B.P.

Dilute 1 ml. with 2 ml. of *strong ammonia buffer* and sufficient water to produce 1 litre. Take 10 ml. in a 150 ml. separator and continue as described in the general method (chloroform extraction procedure) beginning with the words "Add 1 ml. of 4-*aminophenazone solution* . . ." but using an Ilford No. 604 filter.

Ointment of Methyl Salicylate B.P.C.

The method described below has given satisfactory recoveries but if the alkaline solution is allowed to stand just before colour development a progressive hydrolysis of the ester occurs and low results are obtained. No serious loss takes place within 5 minutes, however, and this allows adequate time to carry out the necessary operations of mixing, pipetting and adding the aminophenazone and ferricyanide reagents.

To about 0·5 g., accurately weighed, in a 100 ml. round-bottomed flask add 20 ml. of ethanol (95 per cent) and two glass beads; reflux for 30 minutes on a water bath using a straight bore water condenser. Allow to cool and wash down the condenser with 10 ml. of ethanol (95 per cent); cool thoroughly and filter through a fast filter paper (Postlip Mills 11 cm.) into a 100 ml. graduated flask. Wash the flask and paper well with ethanol (95 per cent) to produce 100 ml. Transfer 10 ml. to a litre graduated flask which already contains 2 ml. of strong ammonia buffer and 800 ml. of water and dilute to 1000 ml. with water. Mix thoroughly and immediately transfer 10 ml. to a 150 ml. separator and continue by the general method (chloroform extraction procedure) beginning with the words "Add 1 ml. of 4-*aminophenazone solution* . . ." but using a 0·5 cm. cell.

This method is equally applicable to Compound Ointment of Methyl Salicylate and may be carried out much more rapidly than the official procedure.

Non-staining Ointment of Iodine with Methyl Salicylate B.P.C.

This method is similar to that described for Ointment of Methyl Salicylate except that an additional heating with ethanol has been prescribed since a somewhat intractable residue is formed from which it is difficult to extract all the methyl salicylate with a single treatment.

Proceed by the method described for Ointment of Methyl Salicylate to the words "filter through a fast filter paper (Postlip Mills 11 cm.) into a 100 ml. graduated flask". During this filtration retain the bulk of the residue in the flask. Wash flask and filter two or three times with ethanol (95 per cent), add 20 ml. of the ethanol to the residue in the flask and reflux again for 30 minutes; cool and filter through the same paper. Wash flask and filter with ethanol (95 per cent) until exactly 100 ml. of filtrate and washings have been collected. Transfer 10 ml. to a litre flask which already contains 2 ml. of strong ammonia buffer and 800 ml. of water and dilute to 1000 ml. with water. Mix thoroughly and immediately add 50 ml. to a 150 ml. separator and continue by the general method (chloroform extraction procedure) beginning with the words "Add 1 ml. of 4-*aminophenazone solution* . . ." but using a 0·5 cm. cell.

Gargle of Potassium Chlorate and Phenol B.P.C.

Again a direct application of the general method is possible without the need for the steam-distillation procedure described in the B.P.C. The method described for Solution of Chloroxylenol is applicable except that 2 ml. of sample is taken and an Ilford No. 602 filter is used.

Ointment of Zinc Oxide and Camphor B.N.F.

In this case it has been found convenient to extract the phenol into an alkaline aqueous solution and this is conveniently carried out by the following method:—

Transfer about 0·5 g., accurately weighed, to a 250 ml. round-bottomed flask and add 25 ml. N sodium hydroxide, 5 ml. of industrial methylated

spirit and two glass beads. Reflux for 1 hour under a straight bore condenser; wash down with plenty of hot water and allow to cool. Filter through a fast paper (Postlip Mills 11 cm.) into a litre graduated flask and wash well with cold water. To the contents of the graduated flask add 5 ml. of a 25 per cent solution of ammonium chloride and dilute to the mark. Mix thoroughly, transfer 10 ml. to a 150 ml. separator and continue by the general method (chloroform extraction procedure) beginning with the words "Add 1 ml. of 4-*aminophenazone solution*. . . ".

Carbolised Oil B.P.C. 1949

A similar procedure to that described for the above ointment may be applied, but using 0·5 g. of sample and 12·5 ml. of 2N alcoholic potassium hydroxide.

Determination of Hexachlorophene in a Dusting Powder

The formulation under consideration contained hexachlorophene (1 per cent), together with other organic substances and fillers. The determination is of particular interest since hexachlorophene forms with 4-aminophenazone a dye which is insoluble in chloroform and the aqueous procedure must therefore be used. The following method has given good recoveries.

To about 1 g., accurately weighed, add 20 ml. of acetone and stir well. Allow to settle and decant the supernatant liquid through a filter paper into a dry 100 ml. flask, washing the residue and filter paper with successive quantities of acetone to remove all soluble material. Adjust the volume of the filtrate to 100 ml., mix and transfer 2 ml. into a 50 ml. graduated flask. Continue by the general method (aqueous procedure) commencing with the words "Add 1 ml. of 4-*aminophenazone solution*. . . ".

Determination of Phenol in Strong Solution of Iodine with 2 per cent Phenol

Dilute 25 ml. of the sample to 100 ml. with 90 per cent ethanol. To 10 ml. add 20 ml. of water and titrate with 0·1N sodium thiosulphate until the iodine is just decolourised. This titration may be used to report the percentage of iodine present. Wash the titration solution into a litre graduated flask, add 2 ml. of *strong ammonia buffer* and dilute to the mark. Mix and transfer 5 ml. of the dilution to a 150 ml. separator and continue by the general method (chloroform extraction procedure) commencing with the words "Add 1 ml. of 4-*aminophenazone solution*. . . ".

Determination of p-*Hydroxybenzoates used as Preservatives*

Methyl *p*-hydroxybenzoate is not directly determinable, of course, but a preliminary hydrolysis converts it to the acid and the general method may then be applied. The following procedure has been found to be satisfactory for the determination of methyl and propyl *p*-hydroxybenzoates in a number of preparations.

Take 10 ml. of sample containing 0·2 per cent of methyl *p*-hydroxybenzoate in a 250 ml. conical flask and wash in with 10 ml. of water. Add 25 ml. of N sodium hydroxide solution and reflux gently for 2 hours. Wash down the condenser with water, cool and transfer to a litre graduated flask containing 5 ml. of a 25 per cent solution of ammonium chloride.

Wash the flask out with water and dilute the hydrolysate and washings to 1 litre. Mix and filter, rejecting the first few mls. Transfer 10 ml. of the filtrate to a 150 ml. separator and proceed as described in the general method (chloroform extraction procedure) commencing with the words "Add 1 ml. of 4-*aminophenazone solution*. . . ".

TABLE III

RECOVERY OF PHENOLIC COMPOUNDS FROM PHARMACEUTICAL PREPARATIONS

Preparation	Content of phenolic substance		
	Expected	Found by proposed method	Found by reference method
Solution of Chloroxylenol B.P.†	Chloroxylenol 5·0 per cent w/v	5·00	
" " " *		5·08	5·11
" " " *		5·28	5·08
" " " *		5·00	5·15 (Piria and Schiff)
Carbolised Oil B.P.C. 1949†	Phenol 5·0 per cent w/v	5·12	
" " " "		4·94	4·80 (Bromometric)
Garg. Pot. Chlor. and Phenol B.P.C.†	Phenol 1·30 per cent w/v	1·30	
" " " " " *		1·26	1·24 (B.P.C. Method)
Hexachlorophene dusting powder†	Hexachlorophene 1·0 per cent w/v	0·97	
" " " †		1·03	
" " " †		0·97	
" " " *		1·02	
Ointment of Zinc Oxide and Camphor B.N.F. †	Phenol 3·2 per cent w/v	3·16	
" " " " " *		2·95	3·39
" " " " " *		3·17	3·28 (Iodimetric)
Ointment of Methyl Salicylate B.P.C.†	Methyl salicylate 50 per cent w/v	49·2	
" " " " *		49·4	51·2
" " " " *		52·1	48·5
" " " " *		52·6	48·9 (B.P.C. Method)
Non-staining Ointment of Iodine with Methyl Salicylate †	Methyl salicylate 5·9 per cent w/v	5·83	
" " " " *		5·82	5·72
" " " " *		5·49	5·44
" " " " (old sample)		5·36	5·53 (B.P.C. Method)

* Production samples.
† Laboratory prepared samples.

Table III lists some results obtained in recovery experiments on some of the examples given above. The few applications given have been chosen to demonstrate the scope of the method, since it would not be practicable to list all the applications which have been made.

DISCUSSION

The 4-aminophenazone method is considered to be superior to many other methods of determining phenols because it is simple to operate, it is rapid, the results are reproducible, the reagents are readily available and are stable, and the method is applicable over a fairly wide range of concentrations.

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DISCUSSION

The paper was presented by MR. R. A. SAVIDGE.

THE CHAIRMAN. The method was not of universal application and each preparation would require individual study. Would the sesamol present in sesame oil react with 4-aminophenazone? Did Tubocurarine react?

MR. S. G. E. STEVENS (London). After what time did the change in the dye become appreciable? Was there degradation at the interface during extraction and would this interfere? What was the partition ratio? Could highly emulsified products be separated within 15 minutes by the technique described.

DR. W. MITCHELL (London). Did vanillic acid give a positive reaction?

PROFESSOR W. H. LINNELL (London). Had the authors any evidence that the sulphonic group and all the alkoxy groups were actually eliminated or was this assumed from the production of a colour? There might be another type of reaction with vanillin; the aminophenazone would react with the aldehyde group.

DR. R. E. STUCKEY (London). Had amounts of less than 0·1 per cent of phenol or particularly of hexachlorophene been determined?

DR. F. L. ROSE (Macclesfield). The mechanism of the reaction seemed to be similar to the coupling of diazonium salts which displaced sulphonic acid groups and the same would take place here. Had aniline interfered with the reaction?

MR. H. B. HEATH (Sudbury). Was it necessary to determine a standard curve on each occasion? What instrument was used? Would the results for methyl salicylate using a No. 604 filter have been as reproducible as those using a No. 602 filter?

MR. G. J. W. FERREY (Manchester). Were the results in Table III single determinations or means? Did the Authors suggest that the 7 per cent discrepancy between the methods for ointment of methyl salicylate mean that the B.P.C. method was 7 per cent in error? It would be preferable to dissolve ammonium chloride in water and add ammonia when making the buffer.

MR. C. A. JOHNSON replied. He could not explain why there was apparently no *ortho* coupling and asked assistance from organic chemists for an explanation.

MR. SAVIDGE replied. One could predict if a phenol would react by its structure. If the *para* position were free or occupied by the radicals mentioned in the paper it would react. Stilboestrol had the position blocked so phenol could be determined in its presence. He had not tried sesame oil, tubocurarine or vanillic acid, but no interference had been observed when vanillin was present. It was difficult to believe that the sulphonic acid or chlorine groups were expelled in the reaction, but it was the only theory they could put forward. The results seemed to show that the reaction must take place in the *para* and not the *ortho* position. They had not tried determining hexachlorophene at 0·1 per cent concentration, but many of the phenols in injections had been determined at this level. Aniline interfered with the reaction, but in none of the preparations examined were there any aniline derivatives. Standard curves for each phenol had been prepared. Filter instruments had been used because they were more widely available, but spectrophotometers could be used. The 604 filter had not been used for salicylates. They were investigating the determination of two phenols together by the two-point method. The results in Table III were the means of duplicates, and those who had used the B.P.C. method would understand the discrepancy.

VISCOSITY STUDIES WITH PHOSPHATIDE SOLS

By I. L. Thomas and L. Saunders

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The viscosities of mixed lecithin-lysolecithin sols pass through a high maximum as the relative proportions of the two phosphatides is varied, although sols of the separate components are found to have viscosities differing little from that of water[1]. We have studied this effect further.

Preparations. Lecithin and lysolecithin were prepared from egg yolk[1]. The mixed sols, containing a phosphatide weight fraction of lecithin less than 0·6, were prepared by weighing the dried solids obtained by evaporating to dryness under reduced pressure stock solutions in ethanol of the two materials. The solid mixture was dissolved in a minimum amount of warm ethanol which was then evaporated at 15 mm. pressure to leave a film of intimately mixed phosphatides. This was dispersed to give a clear sol by shaking with warm distilled water. Traces of electrolytes were removed by passing the sol through a column of mixed ion exchange resins.

Sols containing a weight fraction of lecithin greater than 0·6 were made by mixing lysolecithin sols obtained by dissolving the dried solid in warm water, with lecithin sols prepared as follows. A weighed quantity of dry lecithin was dissolved in a small volume of ether; to this, distilled water was added in successive small portions, with vigorous shaking. A smooth white emulsion of lecithin was at first formed which, with more water, became a thick gel and then a translucent sol. Nitrogen was then bubbled through to remove the ether and the sol was out-gassed on a filter pump. Finally, it was passed over mixed ion exchange resins and made up to volume with distilled water. This method could not be used in the presence of lysolecithin, because of the formation of stable foams during the nitrogen bubbling stage.

Viscosity studies with a capillary viscometer at 25°. *Effect of ageing.* Ageing of the sol had a considerable effect on the mixed sols of high viscosity. A sol of lecithin weight fraction 0·4 and total phosphatide content 0·4 per cent (w/v) had a relative viscosity of 10·0 one hour after preparation; this fell to 4·1 after 5 hours, 3·1 after 15 hours, and to a constant value of 3·0 after 24 hours. In view of these results, subsequent measurements were made with sols which had been aged for 24 hours.

Effect of lecithin weight fraction. A series of sols were prepared which contained 0·4 per cent (w/v) total phosphatides with varying weight fractions of lecithin. The results obtained were similar to those described previously[1], with a maximum relative viscosity of 3·22 at a lecithin weight fraction of 0·45. They are summarised in Table I.

Effect of total phosphatide concentration. The viscosities of the sols in which the total phosphatide concentration was varied between 0·1

and 1 per cent at fixed lecithin weight fractions of 0·2, 0·4 and 0·6 are shown in Table II.

TABLE I

RELATIVE VISCOSITIES OF LECITHIN-LYSOLECITHIN SOLS AT 25°

γ	η	γ	η
0	1·02	0·580	1·92
0·20	1·11	0·600	1·37
0·325	1·62	0·650	1·31
0·375	2·87	0·800	1·22
0·449	3·22	0·900	1·17
0·500	2·43	1·000	1·11
0·550	2·20		

Total phosphatide concentration, 0·4 per cent w/v.
γ = weight fraction of lecithin; η = relative viscosity.

TABLE II

VARIATION OF VISCOSITY WITH TOTAL PHOSPHATIDE CONCENTRATION AT 25°

	$\gamma = 0{\cdot}2$			$\gamma = 0{\cdot}4$			$\gamma = 0{\cdot}6$		
C	$10^3\phi$	η_{sp}	η_{sp}/ϕ	$10^3\phi$	η_{sp}	η_{sp}/ϕ	$10^3\phi$	η_{sp}	η_{sp}/ϕ
0·10	0·92	0·05	54	0·93	0·28	303	0·93	0·11	118
0·21	1·93	0·05	26	1·94	0·66	340	1·95	0·28	144
0·31	2·85	0·08	28	2·87	1·06	370	2·89	0·50	173
0·44	4·04	0·14	35	4·07	1·78	437	4·10	0·83	203
0·58	5·24	0·16	31	5·27	3·11	590	5·31	1·78	335
0·69	6·34	0·19	30	6·38	4·06	636	6·42	3·44	535
0·83	7·63	0·22	29	7·68	5·67	738	7·73	5·50	712
1·00	9·19	0·28	30	9·25	55·2	5970	9·31	58·2	6250

γ = weight fraction of lecithin; c = total phosphatide concentration in g./100 ml. of sol.; η = relative viscosity; $\eta_{sp} = \eta - 1$; ϕ = volume fraction of total phosphatide.

These results are interesting because they give some idea of the shape of the macromolecules present. Values of specific viscosity $\eta_{sp} = \eta - 1$ are shown in Table II together with η_{sp}/ϕ where ϕ is the volume fraction occupied by the total phosphatide in the sol.

For the sols of lecithin weight fraction 0·2, the specific viscosity is low and apart from the first two results where the experimental error in η_{sp} is high, the value of η_{sp}/ϕ is independent of total concentration, having a mean value of 30. These results indicate that the macromolecules in these sols of low lecithin content are spherical or very nearly so, a conclusion which is supported by our diffusion measurements. The high value of η_{sp}/ϕ compared with Einstein's theoretical value of 2·5, suggests that the aggregates are hydrated.

With sols of lecithin fraction 0·4, the viscosity-concentration relationship is completely different. The specific viscosities are high and rise sharply with increasing concentration. The values of η_{sp}/ϕ vary with concentration, the plot of η_{sp}/ϕ against ϕ is linear for the more dilute solutions and can be extrapolated to give an intrinsic viscosity $|\eta|$, where $|\eta| = (\eta_{sp}/\phi)_{\phi=0}$ of 274.

The results for the sols of lecithin weight fraction of 0·6 are similar to those of fraction 0·4, the extrapolated intrinsic viscosity being 93 in this case.

The high viscosities of the sols with lecithin fractions of 0·4 and 0·6 and the variation of their values of η_{sp}/ϕ with ϕ, indicate that highly

asymmetric macromolecules are present in these sols. Application of the Simha equation[2], for the intrinsic viscosity of sols containing ellipsoidal particles indicates that if the macromolecules are rod-like entities, their axial ratios (length/diameter) would be 65 and 34 for the sols having lecithin fractions of 0·4 and 0·6 respectively.

If, as is more likely with these phosphatides which readily form film-like structures, the macromolecules are present as plates, then application of another form of the Simha equation[2] gives values of the ratios, plate diameter/plate thickness, of 404 and 137 for these two sols.

The above results are deduced without taking into account the undoubted hydration of the macromolecules, for example 30 per cent hydration would reduce the ratios given above by about 15 per cent for rod-like macromolecules and 25 per cent for plates. It will be interesting to see how these conclusions about particle asymmetry agree with studies of the sols by other physical methods. The decision whether the particles are in fact rod-shaped or disc-shaped cannot be made on the basis of the information available.

Measurements with a cone and plate viscometer at 25°. The apparent relative viscosity of the thick sols, measured by means of an Ostwald viscometer, was found to vary somewhat with the diameter of the capillary. In order to investigate the effect of rate of shear on the apparent viscosity, some measurements have been made with a Ferranti-Shirley cone and plate viscometer with which the shear rate could be varied between 20 and 20,000 sec.$^{-1}$.

A highly viscous sol was used for this study, having a lecithin fraction of 0·4 and a total phosphatide concentration of 2 per cent. Shear stresses at varying shear rates were measured. The plot of stress against rate showed that the viscosity was non-Newtonian at low rates but became Newtonian, with a linear relationship between stress and rate, at rates above 500 sec.$^{-1}$. The apparent relative viscosity was 35 at the lowest shear rate measured, 84·5 sec.$^{-1}$, and fell to a constant value of 4·2 at shear rates above 500 sec.$^{-1}$.

TABLE III

EFFECT OF TIME INTERVAL ON IMMEDIATE SHEAR STRESS OBTAINED IN A SECOND SHEARING OF A VISCOUS SOL, AT 25°

t	S	t	S
0	154	5	129
1	80	6	135
2	86	7	142
3	117	8	154
4	123		

Total phosphatide content of sol, 2 per cent w/v; weight fraction of lecithin, 0·4. Each shearing made at a rate of shear of 84·5 sec.$^{-1}$, for 2 seconds.
t = time interval in minutes between two shearings.
S = immediate shear stress in dynes cm^{-2}.

The change in the apparent relative viscosity on increasing the shear rate may be due to the large asymmetric particles present in the sol becoming orientated in the direction of flow, or it may be due to the

fact that the association of micelles is quite loose and can be broken down to a large extent mechanically. The thixotropic nature of the sol was established by applying a low shear rate for 2 seconds to the sol at varying time intervals and observing the immediate stress produced. The results are shown in Table III. It is seen that at short time intervals, low stresses are obtained at the second shearing, some of the structure in the sol having been broken down by the first shearing. With longer time intervals, however, this structure is able to re-form and with an 8 minute interval, a stress equal to the immediate stress obtained in the first shearing, was found.

We thank Professor E. Shotton for the use of the cone and plate viscometer.

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DISCUSSION

The short communication was presented by DR. L. SAUNDERS.

THE CHAIRMAN. It might be helpful if the solutions were examined in polarised light between polarised screens. Had any progress been made with the purification of the phosphatides? Were they satisfied that the lysolecithins had the α and β structures quoted?

DR. F. HARTLEY (London). It was intriguing that after great precautions to remove electrolytes the proportion of lecithin and lysolecithin giving the highest relative viscosity was nearly equimolecular. Was this profoundly affected by electrolytes?

DR. SAUNDERS replied. He had emphasised throughout that these preparations must be regarded as fractions. The samples were not the same as those used last year. There were variations in the physical properties of some of the batches but the viscosities did not vary greatly. It was not possible to obtain the products as single pure substances. The effect of electrolytes was the next step, but preliminary results suggested that the effect would not be great. He was not so sure that the equimolecular ratio of lecithin and lysolecithin was very significant.

VEGETABLE PURGATIVES CONTAINING ANTHRACENE DERIVATIVES

PART X. A NEW ACTIVE GLYCOSIDE OF SENNA

BY J. W. FAIRBAIRN, C. A. FRIEDMANN AND H. A. RYAN

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Evidence is produced to show that the sennosides are probably breakdown products from primary glycosidal compounds occurring in the crude drug. Carefully prepared extracts have been shown to contain these primary compounds and one such compound has been isolated in a pure form. Biological and chemical assays show it is about 50 per cent more active than the sennosides and the relation of this fact to the explanation of the total activity of senna is discussed.

IN an earlier paper in this series Fairbairn and Saleh[1] have shown that between 60 to 70 per cent of the biological activity of senna pod and senna leaf (*Cassia acutifolia* Delile and *C. angustifolia* Vahl.) can be accounted for by the content of sennosides A and B and a third non-rhein anthracene glycoside present, when these are estimated as sennosides by the method of Fairbairn and Michaels[2]. The remaining 30 to 40 per cent of the activity is due to some unknown factor which could be extracted from the crude drug along with the anthracene glycosides, by hot water and by 70 per cent ethanol, but none of it survived if an aqueous solution was concentrated by prolonged evaporation as directed in the B.P. 1953 for Liquid Extract of Senna[3]. The 30 to 40 per cent activity unaccounted for might be explained by any of the following possibilities. (*a*) The amount of the third glycoside present is larger than reported earlier due to faulty methods of extraction or destructive methods of hydrolysis during assay. (*b*) There is present in the crude drug some non-anthracene compound which exerts a laxative effect. A possibility would be the flavonol glycoside, kaempferin, reported to be present in senna leaf and pod and said to exert a laxative effect[4]. (*c*) The sennosides themselves are breakdown products from primary compounds which may be more active. An analogous situation occurs in such drugs as aconite and digitalis, where fragments of the primary active principles are sometimes broken off during extraction with consequent loss of activity.

The following experiments were therefore designed to investigate these possibilities.

EXPERIMENTAL METHODS AND RESULTS

Preliminary work on the separation and nature of the third (non-rhein) glycoside and its chemical and biological assay, showed that the extra biological activity could not be accounted for by this glycoside since the amounts present in the crude drug were not greater than those previously reported[1]. The first suggested explanation may therefore be ruled out. Of the remaining two explanations that based on the presence of sennoside precursors seemed more likely. Chemical and biological assays of a

large number of samples of senna pod show a close correlation between sennoside content and biological activity[5], which suggests that the latter is associated with the sennosides. To confirm this it was necessary (*a*) to use the mildest condition of treatment possible to minimise breakdown of the postulated primary substance, and (*b*) to separate the acidic glycosides (sennoside-type, based on rhein) from the non-acidic glycosides, such as the non-rhein (third) glycoside and kaempferin. The following further experiments were therefore carried out.

The Preparation of Crude Fractions

Ethanol (70 per cent) extracts of powdered senna pod were prepared in the cold with and without the presence of dilute mineral acid. The rhein glycosides were separated from the non-acidic either by precipitation of the former as calcium salts or by evaporating the ethanol extract to a dry powder in vacuum and removing the non-acidic glycosides from the powder with anhydrous methanol. The rhein fractions from both extracts were then assayed chemically and biologically and the results are shown in Table I.

TABLE I

Analyses of one crude fraction made with neutral ethanol and another with acidic ethanol

Solvent	Chemical assay expressed as mg. sennosides A and B/g.	Biological assay activity equivalent to sennosides A and B (mg./g.)	B/C ratio*
Neutral ethanol (70 per cent)	260	397 (248–466)**	1·5
Ethanol (70 per cent) containing 0·5 per cent HCl	327	385 (313–473)**	1·2

* Biological assay result, expressed in terms of sennosides, divided by chemical assay result expressed in the same terms. With pure sennosides the ratio would be unity.
** Limits of error, P = 0·95.

It will be seen that when acidic ethanol was used for extraction the biological activity was not much higher than would be expected if sennosides only were present in the resulting extract. On the other hand, when neutral ethanol was used the resulting extract was 50 per cent more active than would be expected if sennosides only were present. These experiments therefore indicate that the neutral extracts may contain the sennoside precursors and that the use of mineral acid during extraction and concentration should be avoided. They further confirm the suggestion that the enhanced activity of the crude drug is associated with the sennosides rather than with the non-rhein and flavonol glycosides, which were separated from the fractions tested in the above experiments. The following two procedures indicated beyond doubt that enhanced activity was associated with the sennosides.

Isolation of a New Glycoside

(*a*) By Acetylation

Powdered senna pod (100 g.) was stirred with cold neutral ethanol (70 per cent; 1200 ml.) for 8 hours. After filtration, the cake was washed

with a further quantity (400 ml.) of the same solvent and the ethanolic extract was concentrated to small volume in a rotary vacuum still at below 30° and then to dryness in a vacuum desiccator, the final stages being over P_2O_5. The resulting dry powder (25 g.) was stirred with anhydrous methanol (2 × 200 ml.) which removed chlorophyll, practically all the non-rhein anthraquinone (third) glycoside and the kaempferin. The residue (18 g.) was collected, washed with a little ether and dried in a desiccator after which it was dissolved in pyridine (180 ml.) and acetic anhydride (90 ml.) and heated to 100° for 3 hours. Most of the solvent was removed under reduced pressure and the residue taken up in ethyl acetate, which was washed with a little 0·1 N hydrochloric acid and then with water until it was neutral. The ethyl acetate layer was shaken with several portions of 20 per cent potassium hydrogen carbonate solution in order to extract the acidic (acetylated) glycosides; the alkaline solution was weakly acidified with dilute hydrochloric acid and immediately re-extracted into ethyl acetate. This was then washed with water, dried over anhydrous sodium sulphate and concentrated to small bulk (35 ml.).

The ethyl acetate solution was transferred to a silica gel column and eluted with a benzene-ethyl acetate mixture (3 : 1) until the eluate was colourless. This fraction contained the bulk of the acetylated primary glycosides originally present in the dry extract of the pod. Further elution of the column with mixtures containing various proportions of benzene and ethyl acetate yielded small amounts of anthraquinone compounds which remain to be investigated. The main fraction was evaporated to dryness *in vacuo* and dissolved in about 30 ml. of warm ethyl acetate. Light petroleum (40 to 60°) was cautiously added till the solution became turbid. It was then warmed till clear and allowed to cool slowly until crystallisation set in. The crystals were dissolved in the same solvent and treated by the above chromatographic procedure and re-crystallised, until no further changes occurred in the ultra-violet spectrum.

This acetyl substance was shown to be different from acetyl sennoside A and acetyl sennoside B by ultra-violet and infra-red spectra, anthraquinone assay and equivalent weight determinations. The latter gave a value of 960 which indicates that the new substance is a larger molecule than sennoside. It is hoped to publish further details later. Biological assays showed that neither this new acetyl compound nor the acetyl sennosides had any purgative activity. The new acetyl compound was therefore de-acetylated by means of potassium hydroxide in methanol. The resulting product was washed with butanol to remove any unchanged acetyl glycoside but further purification, by removal of excess potassium acetate, was not attempted as this substance would not be likely to interfere with the chemical or biological assays. The usual chemical assay process[2] was carried out on this product which behaved in the process exactly like the sennosides and gave a figure of 46 mg./g., as sennosides. Biological assay, however, showed it had an activity equivalent to 92·8 mg. of sennosides per g. This work therefore showed that senna pod contains a rhein-type glycoside whose acetyl derivative

differs markedly from those of the sennosides and whose biological activity is considerably greater than that of the sennosides. An attempt was therefore made to isolate this material by a more direct method.

(*b*) *By Direct Extraction*

Powdered senna pod (100 g.) was extracted with ethanol (70 per cent) as described in (*a*). The combined ethanolic extracts were stirred with 4 g. anhydrous calcium acetate for 15 minutes. Precipitation of calcium salts was completed by the addition of 1700 ml. of acetone and the combined calcium salts were collected and dried in a vacuum desiccator. The product (10 to 15 g.) was dissolved in water (150 to 200 ml.) and stirred with Zeo-Karb 225 (H^+ cycle; about 20 to 30 g.) until the solution was free from metal ions. The aqueous solution, which contained practically all the primary glycosides initially present, was filtered from resin and evaporated below 30° under reduced pressure to about 20 ml. *iso*Propanol (100 ml.) was added and the resulting yellow precipitate collected and dried over P_2O_5 at room temperature. It was then dissolved as completely as possible in 85 per cent *iso*propanol (150 ml.) and the solution transferred to a cellulose column, which was eluted further with 85 per cent *iso*propanol until the eluate was colourless. The latter was concentrated under reduced pressure to a syrup and *iso*propanol (4·5 volumes) added. The resulting yellow precipitate was recrystallised several times from 90 per cent *iso*propanol. The following is a summary of its properties and differences from the sennosides.

Properties of the New Glycoside and Differences From the Sennosides

Physical Properties

The new glycoside is dull yellow and very soluble in water; in contrast, both sennosides A and B are bright yellow and only slightly soluble in water. It does not crystallise from aqueous acetone or aqueous 2-ethoxyethanol as used by Stoll and co-workers for the sennosides[6].

Physical constants. *M. p.* 157 to 159° (sennoside A decomposes at 200 to 240°, sennoside B melts at 180 to 186°)[8].

Equivalent weight. By potientiometric titration in 80 per cent ethanol, about 620 (sennosides 432).

Molecular weight. Determinations by the isopiestic method using sennoside A as reference substance and 70 per cent ethanol as solvent gave a molecular weight of 1164 (sennosides 862).

Chromatography

Ascending paper chromatography using 85 per cent *iso*propanol as running solvent gave an R_F value for the new glycoside of 0·9 to 1·0, whereas the sennosides remained at the origin.

Hydrolysis and Chemical Assay

When a methanolic solution containing a trace of mineral acid is warmed at about 60° for 10 to 15 minutes and allowed to cool overnight

yellow crystals are formed. These can be recrystallised from 50 per cent acetone and shown to be sennoside by comparison with authentic material.

When hydrolysed and assayed by the normal method[2] the final red solution had an absorption curve the same as that produced by the sennosides when treated similarly. The extinction coefficient of the new glycoside, however, was only 74 per cent of that of sennoside; this corresponds to a sennidine content of 49·3 per cent (sennoside affords 62·5 per cent sennidine).

Chemical and Biological Assays

The results recorded in Table II show that the new glycoside is about 50 per cent more active than the sennosides.

TABLE II

CHEMICAL AND BIOLOGICAL ASSAYS OF THE NEW GLYCOSIDE

Chemical assay expressed as sennosides A and B (mg./g.)	Biological assay activity equivalent to sennosides A and B (mg./g.)	B/C ratio*
743	1130 (1035–1233)	1·52
	Limits of error, P = 0·95.	

* See note to Table I.

DISCUSSION

The total activity of senna pod. These experiments suggest that the sennosides are break-down products of primary glycosides. It has not yet been established whether sennoside A and sennoside B are derived from two separate primary glycosides or from one, but sufficient evidence has been produced to show that at least one primary glycoside is present and that it is about 50 per cent more active biologically than the sennosides (when compared on the basis of sennoside content). If it can be shown that the bulk of the rhein glycosides of senna pod are of this primary type then the remaining 30 to 40 per cent activity referred to in the Introduction will be largely accounted for. Hence the total activity of senna pod could be accounted for by the presence of these primary glycosides together with small amounts of secondary substances and of non-rhein anthracene glycosides. Methods for the chemical estimation of these various compounds are being investigated in order to establish these suggestions on a quantitative basis.

Senna extracts on storage. It has been reported that certain senna extracts, on storage, lose biological activity more rapidly than is revealed by the chemical assay process. This could be explained by assuming that the primary compounds are slowly changed into less active secondary glycosides, which would nevertheless give the same chemical assay figure. Experimental evidence for this assumption has been collected and will be presented at a later date. It has been observed that dry powdered senna pod on the other hand, retains its biological activity over many years.

Acknowledgements. We would like to thank Mr. J. H. Davey for assistance with the biological assays.

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DISCUSSION

The paper was presented by DR. C. A. FRIEDMANN.

THE CHAIRMAN. In view of the isolation of the new primary glycoside was the synergistic hypothesis still accepted? He would have liked to have seen figures for the elementary analyses of the new glycoside. Did the Authors conclude that the chemical assay of senna was useless for judging the biological potency?

DR. W. MITCHELL (London). If the neutral alcohol extract contained the postulated more active primary glycoside why was its biological activity not greater than the extract made with acidified alcohol? Why did the two crude products give essentially the same chemical assay? Did the acidified ethanol extract contain additional biologically inert anthraquinone derivatives? Was the new glycoside crystalline?

MR. C. A. JOHNSON (Nottingham). Were the Authors satisfied that the glycosides obtained by the gentle and the drastic extractions were identical? What were the limits of error in the biological assays? If 600 mice were used, it would probably be ± 15 per cent when the B/C ratio of 1·2 might not be significantly different from 1 and if less animals were used it might not be significantly different from 1·5.

DR. J. B. STENLAKE (Glasgow). The acetylation procedure would not harm the glycosides. He suggested the use of dry ammonia-methanol which would rapidly hydrolyse acetyl groups to give chloroform-soluble acetamide.

DR. J. M. ROWSON (Ibadan). Was Alexandrian or Tinnevelly pod used and had the Authors compared the two varieties? Had chromattographic methods been tried for the estimation of the primary glycoside? Was this considered to resemble either sennoside A or B with two glucose molecules attached, which would give a difference in molecular weight?

DR. FRIEDMANN replied. Synergism still prevailed with the crude primary glycoside. Details of the carbon and hydrogen figures were available and the exact character of the sugar was being investigated. They were also working on another chemical assay which they hoped would enable them to dispense with the biological assay. Their present chemical assay could not be used to determine the biological activity of senna. The material was obtained in a crystalline form from 90 per cent

*iso*propanol. The acetylation technique had been shown by Muhlemann not to affect complicated glycosides. The main work was carried out on Alexandrian pod, but had been repeated on other varieties and on leaf. Only preliminary experiments had been carried out with chromatographic methods. It was fairly certain that there were extra glucose molecules attached and these would account for the increased molecular weight.

DR. FAIRBAIRN replied. The explanation of why the two extracts varied chemically was that the use of an acid solvent gave a product with less inert material; the glycosides were therefore present in higher concentration. The chemical assay was obviously the correct base-line to use in examining these extracts; where the biological potency was higher than would be expected from the chemical assay, it is reasonable to assume that there is a difference in the glycosides present. His experience of the biological assay process would lead him to expect the mean of four assays to vary by not more than $\pm$ 10 per cent from the true mean. The first column of Table II records the chemical assay, expressed as sennosides A and B, as 743 mg./g. If the glycosides were pure sennosides and if four biological assays were made, the mean should fall between 670 and 820 mg./g. The figure shown in the last column was based on four biological assays and it will be seen that the mean and limits of error 1035 to 1233 show a significantly higher value for potency.

ANATOMICAL STUDIES IN THE GENUS DIGITALIS

PART I. THE ANATOMY OF THE INFLORESCENCE OF *D. purpurea* L.

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The morphology and detailed anatomy of the inflorescence of *Digitalis purpurea* have been described. The diagnostic characters which are the most valuable in identifying the inflorescence are the glandular trichomes, which are present on most parts; the pollen grains; the striated cuticle of the calyx and pedicel; the pericyclic fibres of the calyx, pedicel and stem, also the lignified cells of the anther, fruit wall and seed coat.

FLORAL organs have been found in commercial samples of the leaves of *Digitalis purpurea* and *D. lanata*, and these organs must be regarded as foreign organic matter. Since no adequate description of the anatomical characters of the flowers of any *Digitalis* specie could be found, it was decided to make a detailed investigation of the structure of the inflorescence of a number of the more commonly occurring species in the genus. In this way the means may be provided whereby such structures can be identified in crushed or powdered leaf samples. This paper deals with the anatomical structure of the inflorescence of *D. purpurea* L.

Although no description of the anatomy of the flowers of *D. purpurea* could be found, certain descriptions of their morphology have been made[1–12]. These vary considerably in their completeness, and to some extent in their accuracy. Morphological characters, additional to those mentioned in these descriptions, together with details of size, are mentioned under "Gross Morphology".

Material

The inflorescences used for this study were those from several clones grown in the Experimental Gardens of the Museum Department of the Pharmaceutical Society, Birdsgrove House, Mayfield, Derbyshire, also inflorescences from wild plants growing in Sussex and North Wales. In each case the mature flowers possessed all those characters of floral morphology accepted as typical of *Digitalis purpurea* L.

Experimental Methods

At all times chloral hydrate proved to be a satisfactory clearing agent for surface preparations. In order to prepare sections the dried material was soaked in a solution of chloral hydrate containing 8 g. of chloral hydrate in 5 g. of water, for 4 to 6 hours at 40 to 50°. The material was then washed in several changes of distilled water, embedded in polyethylene glycol according to the method of Fell and Rowson[13], and finally sectioned by microtome. Macerates were also prepared using Schulze's maceration fluid and solution of potassium hydroxide.

Gross Morphology

The shape of the sepals was not found to vary with the location of the calyx upon the inflorescence axis and this finding is not in accord with that of Clapham, Tutin and Warburg[5]. The lateral sepals are ovate, 0·6 to 2·2 cm. long and 0·3 to 1·3 cm. broad; the posterior sepal is lanceolate and reduced, 0·5 to 1·2 cm. long and 0·3 to 0·5 cm. broad (Fig. 1, A). The sepals become reflexed as the fruit matures (Fig. 6, B). Each corolla lobe has a slightly recurved margin and 3 to 4 main veins, secondary and tertiary veinlets are present in the distal region only (Fig. 3, A, G). The corolla tube is 1 to 3 cm. in diameter at the distal end.

The androecium consists of four didymous stamens. The filaments are glabrous, curved, about 1 mm. wide. Each member of the posterior-lateral pair is 14 to **18** to 21 mm. long and the members of the anterior-lateral pair are 20 to **24** to 26 mm. long. The length of the adherent portion of the filament, 10 to **11** to 13 mm. for the posterior-lateral pair and 8 to **10** to 12 mm. for the anterior-lateral pair, varies inversely with the total length of the filament[14]. The didymous anther lobes are glabrous, conical, with convex sides, 1·75 to 2·25 mm. long and about 1 mm. wide at their base. Dehiscence is introrse, the split extending the entire length of the lobe (Fig. 4, A and H).

The yellowish-green gynoecium (Fig. 5, A), is 9 to 12 mm. long and 4 to 6 mm. wide at the base. There are about 40 more or less parallel veins in the ovary wall, with very few secondary and tertiary veinlets (Fig. 5, P). The nectary at the base of the ovary consists of four slightly curved portions of tissue, yellow in colour, with their ends closely appressed to the adjacent portions (Fig. 5, A). The firm, erect, glabrous style, 18 to 24 mm. long and 1 to 2 mm. wide, arises from the apex of the ovary. The terminal, glabrous stigma is formed of two slightly diverging lobes (Fig. 5, A and C).

The brownish-green fruit is about 15 mm. long and 9 to 11 mm. wide at the base. Dehiscence is septicidal and loculicidal (Fig. 6, A, B and D).

In most of the seeds[15] which have been examined two or three longitudinal grooves were present. One, indicating the line of the raphe extends the entire length of the seed, whereas the others vary in both length and depth (Fig. 2, E).

Histology

Calyx. The abaxial surface of the sepal is covered by a thin, striated cuticle, the striations tending to radiate from the trichome bases and from the stomatal guard cells (Fig. 1, B). The epidermal cells at the base of the sepal are polygonal, with straight, or slightly wavy, thickened, anticlinal walls (Fig. 1, C). Their measurements[16] are: L, 28 to **51** to 80 μ; T, 20 to **32** to 48 μ; R, 16 to **23** to 30 μ*. Over the remainder of the surface the epidermal cells are polygonal, with thin, wavy walls having

* In recording measurements the letters L, T and R, have reference to the longitudinal, tangential, and radial directions respectively, of the axis 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.

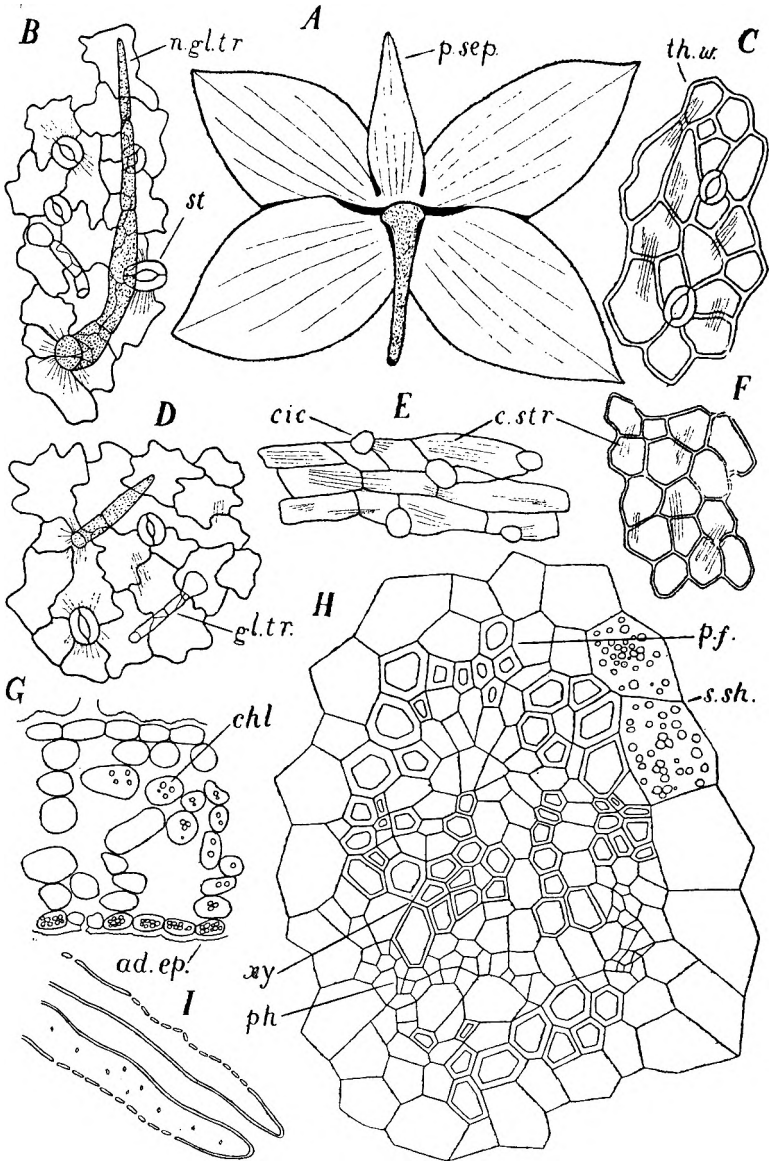

FIG. 1. *Digitalis purpurea* L. Calyx. A, Ventral view of calyx × 1·5. B, Abaxial epidermal cells from apical and central regions. C, Abaxial epidermal cells from basal region. D, Adaxial epidermal cells from central and apical regions. E, Epidermal cells over a vein. F, Adaxial epidermal cells from basal region. All × 150. G, Transverse section of interneural lamina in basal region × 250. H, Transverse section through a main vein × 425. I, Parts of pericyclic fibres, about half of length, × 425. *ad. ep.*, adaxial epidermis with chloroplasts and associated starch grains; *chl*, chloroplasts with associated starch grains; *cic*, cicatrix; *c.str.*, cuticular striations; *gl.tr.*, glandular trichome; *n.gl.tr.*, non-glandular trichome; *p.f.*, pericyclic fibres; *ph*, phloem; *p.sep.*, reduced posterior sepal; *s.sh.*, starch sheath; *st*, stoma; *th.w.*, thickened cellulose walls; *xy*, xylem.

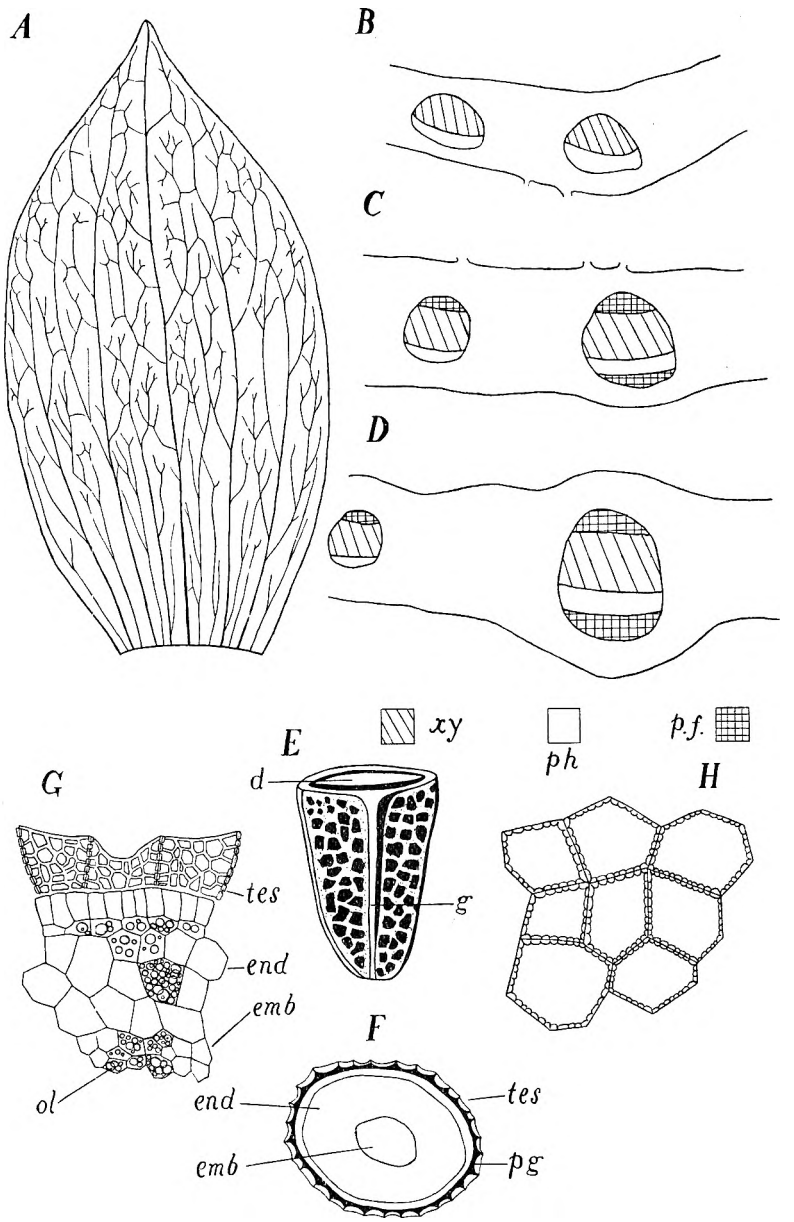

FIG. 2. *Digitalis purpurea* L. Calyx and Seed. A, Sepal, plan of venation × 3. B, C, and D, Tissue plans of transverse sections through mid-rib of sepal in the apical, central and basal regions respectively. E, Seed × 16. F, Tissue plan of transverse section of seed × 50. G, Transverse section of seed × 180. H, Testa in surface view × 180. *d*, depression; *end*, endosperm; *emb*, embryo; *g*, groove marking position of raphe; *ol*, oil globules; *p.f.*, pericyclic fibres; *ph*, phloem; *pg*, pigment; *tes*, testa; *xy*, xylem.

acute re-entrant angles, L and T, 20 to **40** to **60** to 80 μ; R, 16 to **23** to 30 μ (Fig. 1, B). The thin, straight walled epidermal cells over the veins are elongated in the direction of the vein (Fig. 1, E). No starch grains or chloroplasts were found in the epidermal cells. Numerous anomocytic[17] stomata, 28 to **40** to 44 μ long and 18 to **22** to 28 μ wide, are present on this surface (Fig. 1, B and C). The Stomatal Index varies from 3 to **10** to **15** to 22. Abundant trichomes of two types are present on this surface. The non-glandular hairs are multicellular and uniseriate, rarely unicellular, with a slightly warty cuticle, and an acute apical cell (Fig. 1, B). Some of these occur along the edges of the sepal in numbers varying from 12 to 32 per mm. of margin, these are 60 to **248** to 480 μ long, 16 to **26** to 32 μ wide at the base, with 2 to **3** to **4** to 7 cells per trichome. In the interneural areas there are 35 to 60 non-glandular trichomes per sq. mm. which are 40 to **130** to 360 μ long, 16 to **25** to 30 μ wide at the base, and with 1 to **2** to **3** to 5 cells per trichome. The glandular trichomes have a unicellular, or multicellular, uniseriate stalk, with a unicellular, clavate, glandular head. Along each mm. length of margin there are 24 to 32 such trichomes, they are similar in size and in the number of cells per pedicel, to the non-glandular interneural trichomes. In the interneural areas there are 20 to 40 glandular trichomes per sq. mm., 20 to **60** to 200 μ long, 10 to **15** to 20 μ wide at the base, 1 to **1** to **2** to 4 cells per pedicel.

On the adaxial surface the epidermal cells are similar to those on the abaxial side except that the cells in the basal region are less heavily thickened and contain numerous chloroplasts with associated starch grains (Fig. 1, D, F, and G). The stomata also are similar but comparatively rare, the Stomatal Index varies from 0 to **0·2** to **1·7** to 10. Both types of trichome are found on this surface but the non-glandular type is uncommon, and both types are very rare in the basal region. Over the remainder of the surface there are 10 to **60** to 115 glandular trichomes per sq. mm., 20 to **70** to 200 μ long, 12 to **14** to 20 μ wide at the base, with 1 to **1** to 3 to 4 cells per pedicel.

The interneural mesophyll is a loose, lacunose tissue of about five to nine layers of undifferentiated, round to ovoid, or irregularly ovoid parenchymatous cells which contain numerous chloroplasts with associated starch grains, 2 to 8 μ in diameter and possessing a central hilum (Fig. 1, G).

In each sepal the vascular tissue consists of about five main veins, united, in all but the basal fifth, by a network of lateral veinlets (Fig. 2, A). Each main collateral strand is surrounded by a ring of large, rounded cells forming a starch sheath[18], within which is a pericycle[19], consisting of parenchyma and sclerenchyma. The latter is formed of thickened, pitted, sometimes wavy walled fibres of ligno-cellulose, 440 to **560** to 800 μ long, and 8 to **13** to 20 μ wide, with rounded or pointed ends (Fig. 1, H, and I). Their number varies greatly, but generally increases from apex to base (Fig. 2, B-D). The small amount of phloem tissue consists of strands of sieve tubes, each tube being 2 to 4 μ in diameter, together with companion cells and phloem parenchyma. The xylem consists of

a crescent shaped arc of polygonal, spirally and annually thickened, vessels of ligno-cellulose, 4 to **9** to 13 μ wide. The vessels are arranged in files or small groups separated by medullary rays which are one cell in width (Fig. 1, H). The veinlets are not associated with a starch sheath or a pericycle; the xylem is composed of a small group of polygonal tracheids, 4 to **6** to 8 μ wide, with spiral or annular thickening; the very small amount of phloem tissue is somewhat indistinct.

Corolla. The abaxial surface is covered by a thin, smooth, cuticle. The epidermal cells at the base are polygonal, isodiametric, with straight walls, L and T, 6 to **27** to **37** to 60 μ; R, 22 to **30** to 44 μ, this value for R is similar in all regions of the corolla (Fig. 3, B). In the proximal half of the central region the cells have straight walls and are elongated in the longitudinal direction of the corolla, L, 40 to **68** to 100 μ; T, 16 to **25** to 50 μ (Fig. 3, E). Over the remainder of the surface the epidermal cells are polygonal, nearly isodiametric, with wavy walls, very wavy on the free lobes, L and T, 24 to **25** to **50** to 90 μ (Fig. 3, C and D). All the epidermal cells contain simple and compound starch grains; the simple grains are round, 1 to 3 μ in diameter, compound grains of about 4 to 10 components are 8 to 12 μ in diameter, hilum and striations are not visible (Fig. 3, H). Stomata similar to those on the calyx are present on this surface, but are absent from the basal region (Fig. 3, D). The Stomatal Index varies from 1 to **2** to **4** to 7. Trichomes similar to those found on the calyx are present, they are numerous in the distal region and especially so on the free lobes (Fig. 3, C). There are 5 to 10 non-glandular trichomes per sq. mm., 140 to **550** to 2700 μ long, 16 to **23** to 53 μ wide at the base, with 2 to **4** to 7 cells per trichome. The glandular trichomes occur to the extent of 25 to 150 per sq. mm., 60 to **300** to 900 μ long, 16 to **20** to 25 μ wide at the base, with 2 to **4** to 7 cells per trichome. A very few glandular trichomes having a bicellular head with a transverse division were seen.

On the adaxial surface the cuticle and epidermal cells are similar to those of the abaxial side, except that the anticlinal walls of the epidermal cells in the distal region are not so wavy (Fig. 3, F), and the value for R is greater, namely 44 to **55** to 67 μ. Neither stomata nor glandular trichomes were found on this surface, moderately numerous non-glandular trichomes were present but in the distal region only, they have a warty cuticle and an acute apical cell. Their length is 3·4 to 4·7 mm., their width at the base 50 to **70** to 80 μ, the cells per trichome 9 to **12** to 15.

The mesophyll is similar to that of the calyx save for the absence of chloroplasts with associated starch grains, and the presence of anthocyanin and flavone pigments (Fig. 3, H). Ten to 20 main veins are found in the corolla together with secondary and tertiary veinlets. Veins and veinlets are similar except for the size and number of the elements. Starch sheath and pericycle are absent. The xylem vessels, 4 to 10 μ in diameter, with polygonal, ligno-cellulose walls, with spiral or annular thickening, are grouped without obvious arrangement and medullary ray cells are not obvious. The phloem tissue contains sieve tubes 2 to 4 μ in diameter, companion cells and phloem pharenchyma (Fig. 3, I).

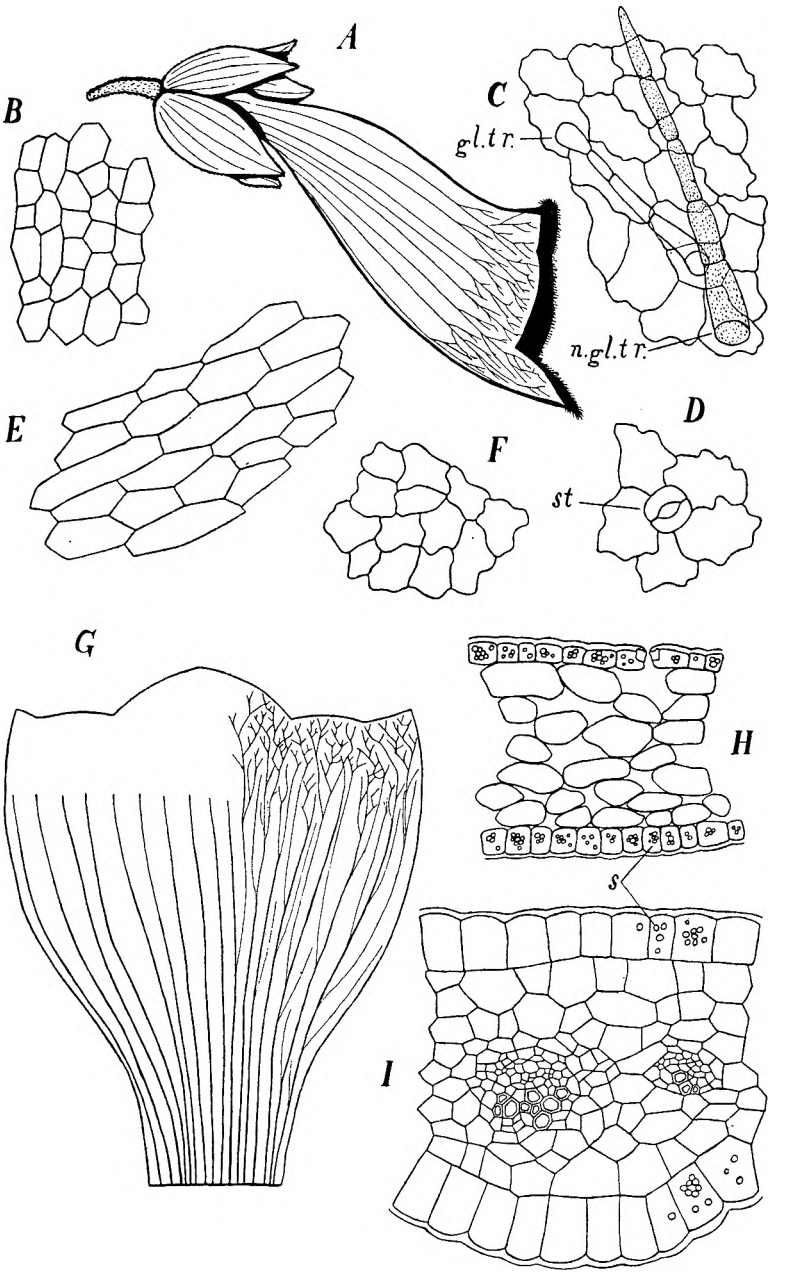

FIG. 3. *Digitalis purpurea* L. Corolla. A, Lateral view of corolla × 1·5. B, Abaxial epidermal cells from extreme proximal region. C and D, Abaxial epidermal cells from distal region. E, Abaxial epidermal cells from central region. F, Adaxial epidermal cells from distal region. All × 150. G, Corolla, plan of venation × 2. H, Transverse section through central region of corolla. I, Transverse section through vascular tissue of corolla, both × 150. *gl.tr.*, glandular trichome; *n.gl.tr.*, non-glandular trichome; *s*, starch; *st*, stoma.

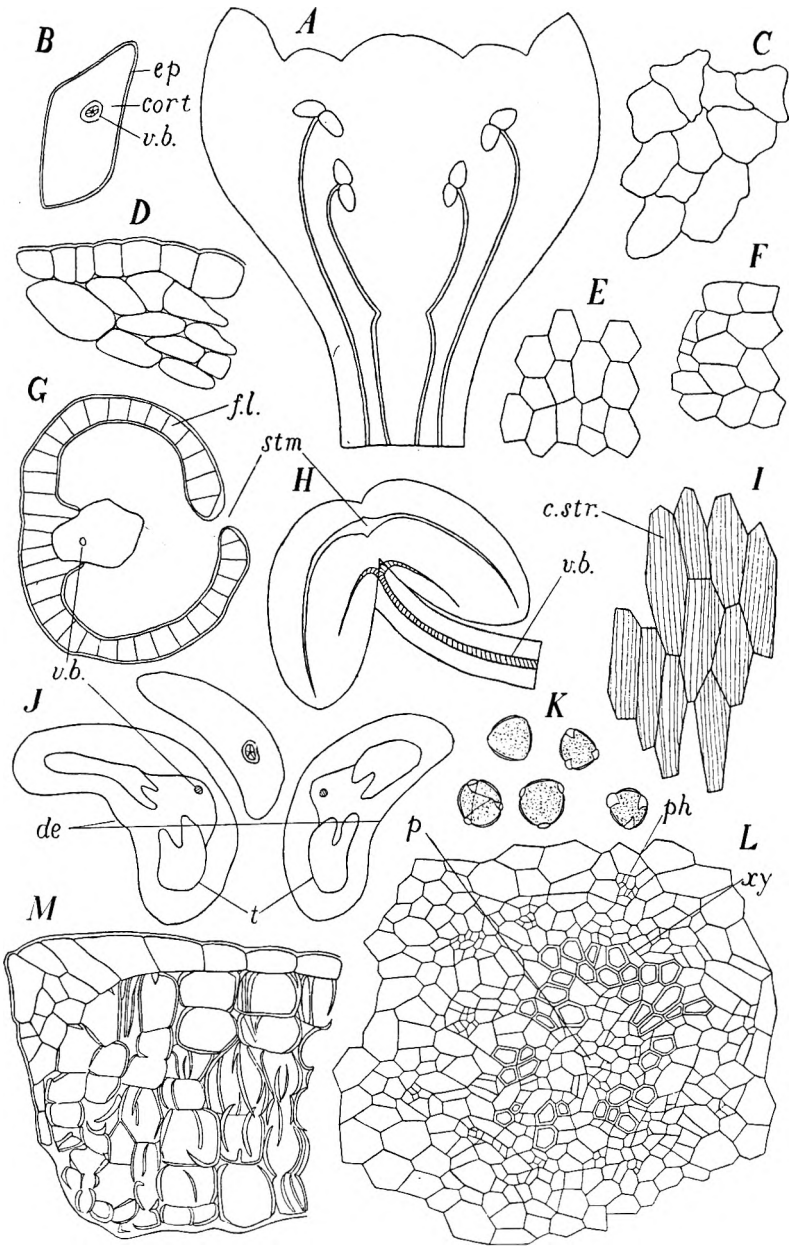

FIG. 4. *Digitalis purpurea* L. Androecium. A, Plan of corolla to show position of stamens × 2. B, Tissue plan of transverse section of filament × 25. C, Epidermal cells of anther lobes. D, Transverse section of filament—epidermis and cortex. E, Epidermal cells of filament near anther. F, Epidermal cells from region of stomium. All × 250. G, Tissue plan of transverse section of anther after dehiscence × 30. H, Anther lobes and filament × 10. I, Epidermal cells of filament from free portion × 250. J, Tissue plan of transverse section of anthers with filament × 25. K, Pollen grains × 250. L, Transverse section of vascular strand of filament × 425. M, Transverse section of anther including stomium × 250. *cort*, cortex; c. *str.*, cuticular striations; *de*, position of dehiscence lines; *ep*, epidermis; *f.l.*, fibrous layer; *p*, pith; *ph*, phloem; *stm*, stomium; *t*, tapetum; *v.b.*, vascular strand; *xy*, xylem.

Androecium. The filament is covered by a thin, uniform cuticle which is striated in the upper third of the free portion, the striations being parallel to the longitudinal axis of the filament (Fig. 4, I). The thin walled, polygonal, almost isodiametric epidermal cells at the base of the adherent portion are L and T, 20 to **48** to **58** to 80 μ; R, 19 to **27** to 34 μ. Over the remainder of the filament the cells are elongated in the direction of the longitudinal axis, L, 90 to **130** to 200 μ; T, 10 to **20** to 30 μ; R, 19 to **27** to 34 μ; this elongation is less apparent in the cells very near to the anthers where the measurements are L, 24 to **32** to 50 μ; T, 12 to **17** to 22 μ; R, 19 to **27** to 34 μ (Fig. 4, E). Neither stomata nor trichomes were found on the filaments examined.

The cortex consists of a loose tissue of 6 to 20 rows of ovoid, or irregularly ovoid, thin walled parenchymatous cells, with numerous intercellular air spaces. Apart from a few chloroplasts in the periphery the cells are devoid of contents (Fig. 4, D). Embedded in the cortex is the eccentric, rarely centric, vascular strand, consisting of a small central pith of partially collapsed cells surrounded by a ring of polygonal, lignified, xylem vessels, 4 to 12 μ in diameter, with spiral and annular thickening. Some medullary ray cells are visible. Surrounding the xylem is a ring of phloem tissue consisting of sieve tubes, 3 to 6 μ in diameter, companion cells and phloem parenchyma. The vascular strand divides in the connective, the two resultant stands supplying the two anther lobes (Fig. 4, B, H and L).

The anther lobes are covered by a thin cuticle, the epidermal cells are polygonal with slightly wavy walls, L, 28 to **50** to 65 μ; T, 20 to **29** to 50 μ; R, 18 to **22** to 30 μ (Fig. 4, C). In the region of the stomium the cells have straight walls and are slightly smaller, L and T, 16 to 24 μ; R, 8 to **12** to 20 μ (Fig. 4, F). No contents were seen in these epidermal cells. Beneath the epidermis there are about five rows of almost isodiametric fibrous cells with ligno-cellulosic thickening in spiral and annular bands, L and T, 20 to **26** to **37** to 50 μ; R, 20 to **36** to 50 μ (Fig. 4, G and M). At the stomium these cells are absent and adjacent to the stomium the number of rows is less, the cells are also smaller, the fibrous tissue therefore forms a pair of slightly tapering blunt pincers which close upon the stomium (Fig. 4, G and M). The fibrous cells adjacent to the stomium are more heavily lignified than the others. Remains of the collapsed tapetum is sometimes visible within the fibrous layer. Stomata and trichomes are absent from the anther lobes.

The pollen grains (Fig. 4, K), are subspherical bodies, 19 to **26** to 32 μ in diameter, with three equally placed germinal pores, 7 to 10 μ in diameter, situated at the widest part of the three germinal furrows which taper towards the poles but do not meet. The intine is smooth, the exine slightly pitted. Starch and oil were not found in the grains examined, in iodine the grains stained a deep yellow.

Gynoecium. The ovary is covered by a thin, smooth cuticle. The polygonal epidermal cells with thin straight walls measure, L and T, 12 to **17** to 29 μ; R, 12 to **18** to 24 μ (Fig. 5, G and H). Anomocytic stomata occur (Fig. 5, G), the guard cells lie in the same plane as the surrounding

epidermal cells. The stomata measure, L, 24 to **30** to 40 μ; B, 20 to **22** to 24 μ. The Stomatal Index has a mean value of 2. Only glandular trichomes occur, they are very numerous and are similar to those of the calyx, 54 to **300** to **450** to 1000 μ long; 12 to **25** to **30** to 60 μ wide at the base; 1 to **1** to **3** to 6 cells per pedicel; 170 to **195** to **230** to 270 trichomes per sq. mm. The cells of the inner epidermis of the ovary are elongated at right angles to the longitudinal axis and have thin straight walls, T, 20 to **55** to 67 μ; L, 5 to **7** to 9 μ; R, 6 to **9** to 12 μ (Fig. 5, M). Neither trichomes nor stomata were found on this inner surface.

The mesophyll is formed of polygonal cells with thin, straight, or slightly wavy walls, some small intercellular air spaces are present. The cells contain chloroplasts with which are associated small, round starch grains of diameter 3 to 5 μ (Fig. 5, O). About 40 vascular strands are embedded in this mesophyll, the polygonal vessels, 4 to **7** to 10 μ in diameter, have lignified thickening of the spiral and annular type; the phloem is composed of very small elements. The cells of the placenta are similar to those of the mesophyll (Fig. 5, K), except that they appear devoid of contents. About eight vascular strands, similar to those of the mesophyll, supply the placenta. Numerous anatropous ovules with one integument are borne on the placenta, when mature these parenchymatous bodies are 12 to **16** to 19 μ long and 10 to **11** to 14 μ wide. The cells of the septum are also similar to those of the mesophyll (Fig. 5, J).

The style is covered by a thin, striated, cuticle, the striations are parallel with the longitudinal axis of the style. The epidermal cells are polygonal, with thin, straight walls and are elongated in the direction of the axis (Fig. 5, N). They measure L, 350 to **450** to 600 μ; T, 16 to **19** to 24 μ; R, 12 to **16** to 22 μ. Beneath the epidermis is the cortex, composed of thin walled cells which surround a small central canal. Two vascular strands are embedded in the cortex, opposite to each other and near to the epidermis (Fig. 5, E), each strand consists of a ring of lignified, polygonal xylem vessels, 3 to 9 μ in diameter; the xylem is surrounded by a ring of very small celled phloem tissue (Fig. 5, I). These two strands supply the two diverging lobes of the parenchymatous stigma (Fig. 5, C).

Fruit. The outer surface is covered by a thick, smooth, cuticle. The polygonal epidermal cells, L and T, 20 to **32** to **49** to 68 μ; R, 17 to **25** to 32 μ, have straight walls and, except for an occasional small rounded starch grain, are devoid of contents (Fig. 6, E). Stomata, similar to those on the ovary wall, are present on this surface (Fig. 6, E). The trichomes are also similar to those on the ovary wall (Fig. 6, E), but they measure only 80 to **220** to 480 μ in length and only 10 to **25** to 40 per sq. mm. The cells of the inner epidermis are similar to those on the inner surface of the ovary wall except that the elongation, T, 68 to **190** to 340 μ; L, 7 to **12** to 17μ; R, 7 to **11** to 14 μ, has become more pronounced (Fig. 6, C). Trichomes and stomata are absent on this inner surface.

The mesocarp (Fig. 6, F), is formed of five to seven rows of cells, the row adjacent to the endocarp is sclerenchymatous, the remainder are parenchymatous. These latter consist of closely packed polygonal, straight walled cells, containing a few chloroplasts. The sclerenchymatous

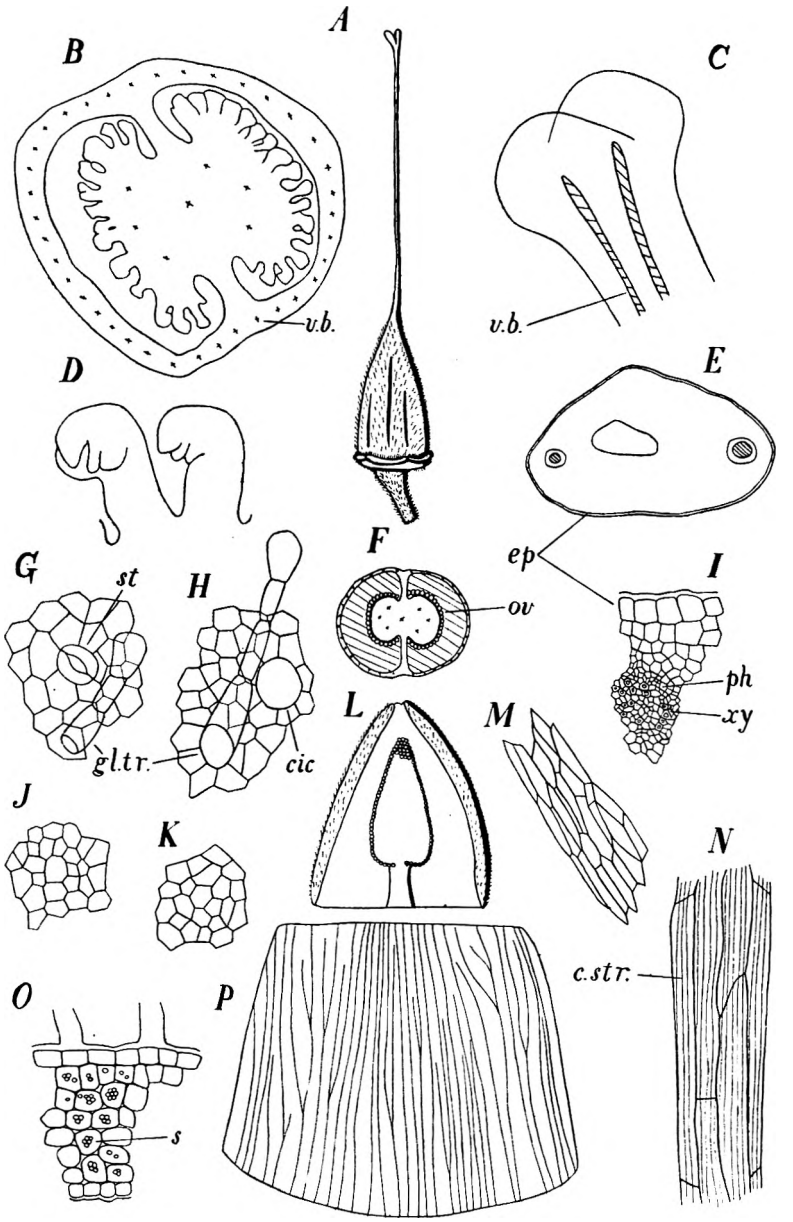

FIG. 5. *Digitalis purpurea* L. Gynoecium. A, Gynoecium × 1·5. B, Transverse section of ovary × 8. C, Stigma with vascular strands × 10. D, Ovules × 100. E, Transverse section of style × 20. F, Transverse section of ovary × 3. G, Outer epidermal cells from central region of ovary. H, Outer epidermal cells from base of ovary. I, Transverse section of style through vascular strand. J, Cells of septum. K, Cells of placenta. All × 180. L, Longitudinal dissection of ovary × 3. M, Inner epidermal cells of ovary. N, Epidermal cells of style. O, Transverse section of ovary wall. All × 180. P, Ovary wall, plan of venation × 5. *cic*, cicatrix; *c.str.*, cuticular striations; *ep*, epidermis; *gl.tr.*, glandular trichome; *ov*, ovules; *ph*, phloem; *s*, starch; *st*, stoma; *v.b.*, vascular strand; *xy*, xylem.

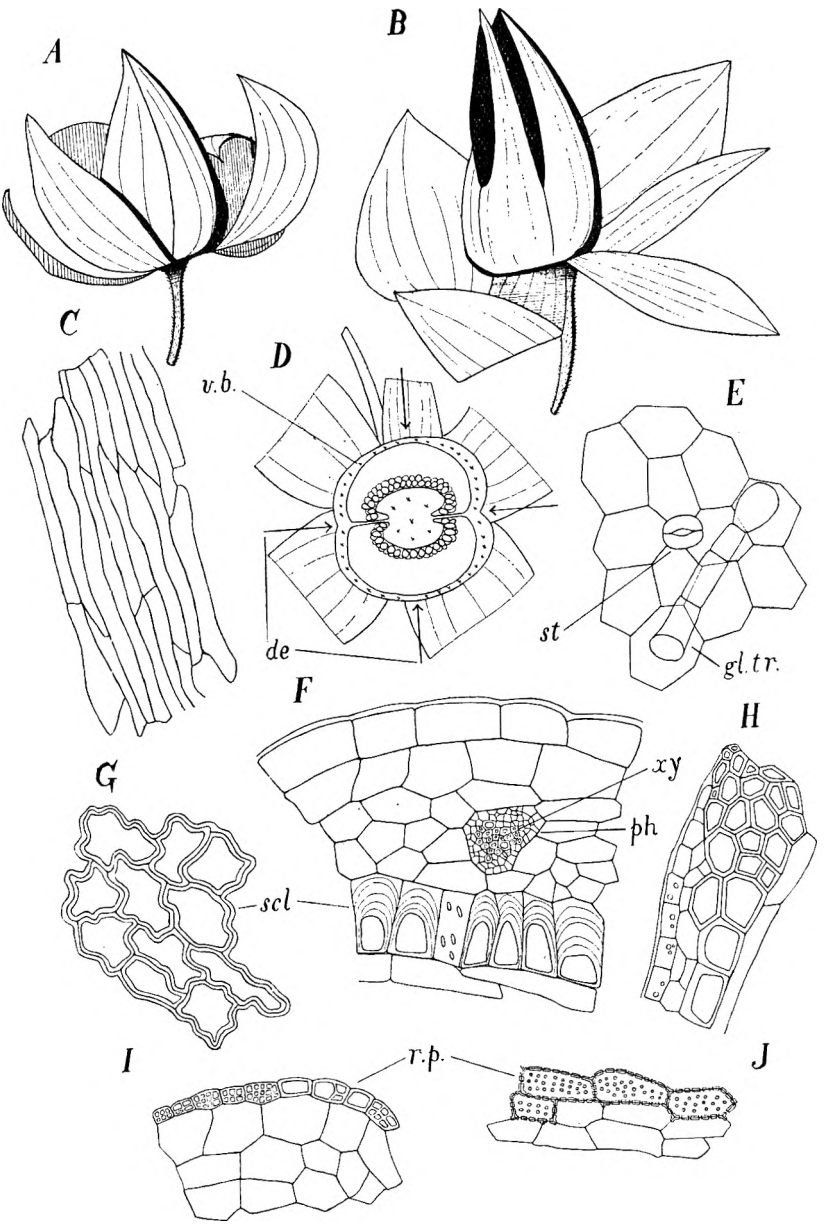

FIG. 6. *Digitalis purpurea* L. Fruit. A, Fruit × 6. B, Fruit showing lines of dehiscence × 6. C, Inner epidermal cells of fruit wall × 180. D, Transverse section of fruit × 6. E, Outer epidermal cells of fruit wall. F, Transverse section of mesocarp. G, Sclerenchymatous layer in surface view. H, Transverse section of mesocarp at point of loculicidal dehescence. I, Transverse section of placenta. J, Covering cells of placenta in surface view. All × 180. *de*, position of dehiscence lines; *gl.tr.*, glandular trichomes; *ph*, phloem; *r.p.*, reticulate parenchyma; *scl.* sclerenchyma; *st*, stoma; *v.b.*, vascular strand; *xy*, xylem.

cells have lignified U-shaped thickening and measure L, 34 to **48** to 69 μ; T, 17 to **26** to 34 μ; R, 20 to **29** to 38 μ; viewed tangentially these cells appear wavy (Fig. 6, G), occasional slit-like pits are visible in the thickening. The vascular strands are similar in all respects to those in the ovary wall. The placenta is also similar, except that the surface has become irregular and covered by reticulate parenchymatous cells (Fig. 6, I and J), which measure L and T, 14 to **20** to **42** to 68 μ; R, 14 to **19** to 22 μ. At the fruit stage the septum is bounded on both sides by the sclerenchymatous layer and inner epidermis. The dehiscence is septicidal and loculicidal (Fig. 6, D). At the septum, splitting occurs in the central parenchyma; the loculicidal splits occur opposite the median part of the placenta, at these points the mesocarp is reduced, the sclerenchyma is wider, and the inner epidermis is absent; the dehiscence occurs in the sclerenchyma.

Seed. The histology of the seed has been described[15], but in addition the following characters have been noted. The epidermal cells measure L, 34 to **59** to 72 μ; T, 30 to **42** to 54 μ; R, 34 to **51** to 64 μ. From the layer of collapsed cells adjacent to these epidermal cells it would appear that a diffuse reddish-brown pigment originates, this spreads over part of the anticlinal walls of the epidermis.

Pedicel. The surface is covered by a thick, striated cuticle, the striations of which are parallel to the longitudinal axis of the pedicel. The polygonal, elongated, epidermal cells have thin and straight, or slightly wavy walls (Fig. 7, E). Their dimensions are L, 36 to **80** to 115 μ; T, 11 to **16** to 21 μ; R, 11 to **15** to 22 μ. Stomata, similar to those on the calyx, are present (Fig. 7, E); the Stomatal Index varies from 3 to **5** to 7. Both non-glandular and glandular trichomes are numerous on all parts of the pedicel, the former, similar in appearance to those on the calyx, are 105 to **320** to 790 μ long, 18 to **27** to 36 μ wide at the base, with 2 to **4** to 6 cells per trichome and 35 to **80** to 120 trichomes per sq. mm. The glandular trichomes, also similar in appearance to those on the calyx, are 70 to **153** to 350 μ long, 11 to **21** to 28 μ wide at the base, with 1 to **3** to 5 cells per stalk and 10 to **30** to 60 trichomes per sq. mm. (Fig. 7, E).

The cortex consists of about eight rows of loosely packed polygonal cells with thin, straight, or rounded walls. The cells are filled with chloroplasts and associated starch grains which are small and rounded, 1 to 3 μ in diameter. The inner periphery of the cortex is delimited by the starch sheath, a ring of subrectangular cells distinct in shape from the cortical cells. The concentration of starch in these cells does not appear to differ markedly from that in the cortex. Within this starch sheath the pericycle occurs as 8 to 10 rows of polygonal, thick walled, lignified fibres, 7 to 24 μ in diameter. They have numerous slit-like pits on their walls, and the outer walls, of those fibres situate on the periphery of the pericycle, are wavy (Fig. 7, H). At the time of the fall of the corolla these fibres are not lignified, the lignification proceeds during the development of the fruit. The pericycle surrounds a narrow band of phloem tissue, with

thin walled sieve tubes 6 to 10 μ in diameter. Cambial tissue is not distinct. The ring of xylem tissue is entirely lignified, the polygonal vessels, with straight and thickened walls, are 10 to 18 μ in diameter. The thickening is of the annular or spiral type. The central pith consists of cells with thin, straight walls and a few starch grains are present. Fibres, single and in groups, similar to the pericyclic fibres, occur in this region (Fig. 7, B and D).

Inflorescence axis. The histology of the inflorescence axis is similar in many respects to that of the pedicel, the differences only will be noted. The cuticle is smooth (Fig. 7, F). Stomata are 36 to **43** to 48 μ long and 25 to **28** to 36 μ wide. The glandular trichomes are 90 to **216** to 450 μ long, 10 to **22** to 43 μ wide at the base, there are 50 to **75** to 140 such trichomes per sq. mm. There are 0 to **8** to 20 non-glandular trichomes per sq. mm. The cortex consists mainly of collenchymatous cells (Fig. 7, C). This is a variable factor, the collenchyma is minimal, two or three rows, at the apex of the young axis, and maximal, occupying the entire cortex, at the base of mature fruit axes. The pericyclic fibres, 11 to **25** to 36 μ in diameter, have thick walls without pits (Fig. 7, C and G). The pith, surrounding a central cavity, consists of large lignified cells with simple pits (Fig. 7, C). The ring of vascular tissue is nearer the periphery of the organ in the axis than in the pedicel (Fig. 7, A and B).

Discussion

The diagnostic characters which are the most valuable in identifying the inflorescence, when in admixture with the leaf, are as follows:

Calyx. Glandular trichomes with a uniseriate, multicellular stalk and a unicellular head: striated cuticle: epidermal cells with thickened cellulosic walls: pericyclic fibres.

Corolla. Glandular trichomes as on the calyx, some of which are very long: anthocyanin and flavone pigments.

Androecium. Elongated epidermal cells with striated cuticle from the filament: fibrous cells from anther wall: pollen grains.

Gynoecium. Glandular trichomes as on the calyx: elongated epidermal cells with striated cuticle from the style: anatropous ovules with one integument.

Fruit. Glandular trichomes as on the calyx: fibrous cells with U-shaped thickening.

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

Pedicel. Glandular trichomes as on the calyx: elongated epidermal cells with striated cuticle: pericyclic fibres.

Inflorescence axis. Glandular trichomes as on the calyx: pericyclic fibres: lignified pith cells.

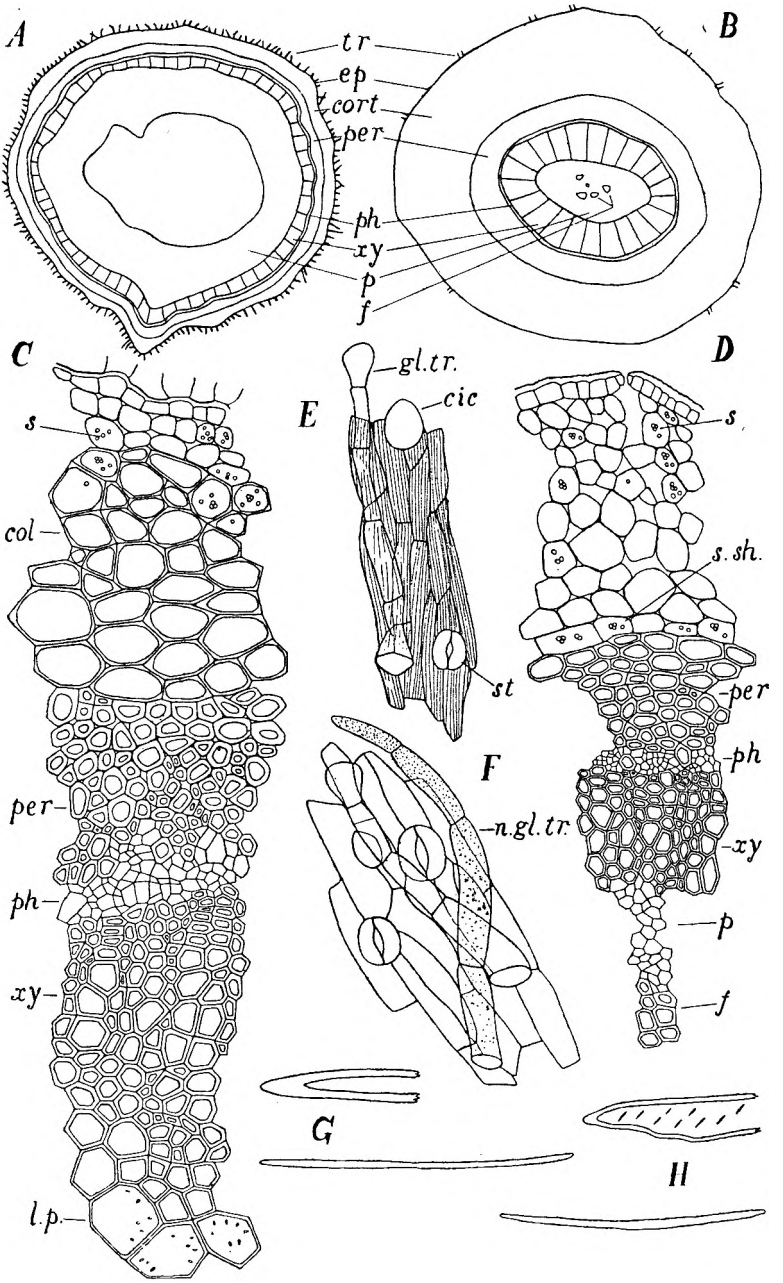

FIG. 7. *Digitalis purpurea* L. Pedicel and Inflorescence Axis. A, Tissue plan of transverse section of inflorescence axis × 8. B, Tissue plan of transverse section of pedicel × 50. C, Transverse section of inflorescence axis. D, Transverse section of pedicel. E, Epidermal cells of pedicel. F, Epidermal cells of inflorescence axis. All × 180. G, Pericyclic fibre from inflorescence axis × 45, and part of same × 180. H, Pericyclic fibre from pedicel × 45, and part of same × 180. *cic*, cicatrix; *col*, collenchyma; *cort*, cortex; *ep*, epidermis; *f*, fibres; *gl.tr.*, glandular trichome; *l.p.*, lignified pith; *n.gl.tr.*, non-glandular trichome; *p*, pith; *per*, pericycle; *ph*, phloem; *s*, starch; *st*, stoma; *tr*, trichomes; *xy*, xylem.

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DISCUSSION

The paper was presented by MR. P. S. COWLEY.

THE CHAIRMAN. It was the experience of some of those handling large quantities of commercial digitalis, that only on rare occasions were flowers present. In the authors experience did the quantity of flowers ever exceed the B.P. permitted limit of 2 per cent of foreign matter? Could the B.P. method for estimating foreign matter be applied and what microscopical characteristics were recommended for determining the inflorescence in the powdered drug?

DR. J. W. FAIRBAIRN (London). There could be no possibility of flowers in digitalis grown in this country, since this is now grown as an annual. Was the material examined by the Authors either foreign or wild? Was the contamination serious? Were there glycosides in the flowers? Was there a microscopic method for estimating the flower in the drug?

DR. T. E. WALLIS (London). Prepared digitalis might contain lucerne which has numerous flowers and the corolla of these would need to be compared with that of digitalis.

MR. V. REED (London). Are there instructions that leaves of commercial samples should be washed?

MR. COWLEY replied. No quantitative estimations of commercial samples had been made. In powders they could detect 0·2 per cent of added powdered corolla in powdered leaf using only one slide. It was possible to detect 0·1 per cent of matured fruits in the leaf. The B.P. did not direct that the leaves should be washed.

DR. ROWSON replied. He agreed that the majority of English digitalis was first-year biennial, continental material was frequently second year.

His experience was that contamination with flowers or mature fruit could easily occur when harvesting second-year biennial. Chemical tests showed the flowers to be moderately active, but not more than the leaf and he believed the glycosides in the seed differed from those in the leaf. No doubt the B.P. Appendix method could be applied to determine the corolla as the epidermis was characteristic. To estimate the entire inflorescence axis would be more difficult. He agreed that the characters of lucerne flowers would have to be investigated before it was possible to determine the amount of inflorescence in Prepared Digitalis B.P.

FACTORS INFLUENCING PERCUTANEOUS ABSORPTION

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The percutaneous absorption of drugs from different vehicles or bases through the intact skin of the rabbit seems to be influenced by a number of factors[1,2].

When sulphanilamide in various vehicles and bases is applied to the intact skin of rabbits, it penetrates the skin and is absorbed into the blood stream where it is detected in measurable quantities[1]. After such a test the blood level falls to zero in four to five days. During the six hours of the test the half-hourly blood samples showed a fluctuation in concentration of sulphanilamide (Table I) although the graph of concentration of drug against time remained approximately parallel. The figures in Table I

TABLE I

Percutaneous absorption of sulphanilamide in the rabbit over six hours
Sulphanilamide observed, mg./100 ml.

Time in hours ..	0·0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	4·5	5·0	5·5	6·0
Average blood level	0·00	0·56	0·75	0·54	0·85	0·46	0·45	0·41	0·77	0·66	0·75	0·36	0·51
Total blood level ..	0·00	3·33	4·48	3·26	5·07	2·73	2·72	2·44	4·59	3·94	4·50	2·14	3·10

give the average blood levels and the total blood levels observed for six rabbits (3 male and 3 female litter-mates) over six hours at half hour intervals. A statistical analysis of the blood levels at half hour intervals showed them to be significantly different. These observations suggested that either absorption or excretion of sulphanilamide occurred in cycles. This phenomenon was noticeable with a number of the other bases or vehicles in which sulphanilamide was incorporated. But when other drugs, like salicylic acid, copper sulphate, and copper acetyl-acetonate were used this rhythmic wave pattern was not seen. It is thought that the explanation of the cycle lies in the tubular resorption of sulphanilamide from glomerular filtration; the acetylated form is not reabsorbed. Since absorption by the percutaneous route is probably continuous, a wave pattern occurs in the blood level.

The physico-chemical properties of the incorporated drugs have been shown to play an important part in percutaneous absorption[2]. Statistical analysis of results showed that the difference in the efficiency as "carriers" of the vehicles or bases tested were significant. But an even more significant result was the interaction between drug and base. From an analysis of all components of the variance the theoretical values may be calculated and compared with those observed. This has been done in Table II for three drugs incorporated in three bases; salicylic acid, copper acetyl-acetonate and copper sulphate in lard, Emulsifying Ointment B.P., and water, as a five per cent carboxymethylcellulose gel. Table II also shows

the per cent deviation of the observed from the calculated results, a positive value gives a result greater than the calculated or theoretical result and a negative the reverse. When the observed value is significantly greater or less than the calculated value an interaction between the particular drug and base is concluded to be in operation. Thus it can be seen that the observed value for salicylic acid when incorporated in lard is higher than the theoretical value as is the observed result for copper sulphate from water. Similarly the copper sulphate : lard result was less than the theoretical value. In these instances, the interaction between drug and base can be considered significant. The variation in the amounts of drugs absorbed from different bases may be accounted for by their physico-chemical properties and an explanation may be offered in terms of their local actions and lipid solubilities.

TABLE II

THE DEVIATION BETWEEN CALCULATED AND OBSERVED RESULTS
DRUGS EXPRESSED IN MG. PER TWO SIX-HOUR TESTS

	Lard			Emulsifying ointment			Water		
	Calculated	Observed	Per cent deviation	Calculated	Observed	Per cent deviation	Calculated	Observed	Per cent deviation
Salicylic acid	206·41	223·43	+8·2	191·84	187·61	−2·2	180·95	168·17	−7·1
Copper acetyl-acetonate	56·17	49·55	−11·8	41·60	43·58	+4·8	30·71	35·37	+15·2
Copper sulphate ..	52·41	42·03	−19·8	37·84	40·01	+6·2	26·96	35·07	+30·0

Sufficient results have been obtained from the series of experiments to analyse for the components of variance. It is a valuable property of variance that if a process has a number of factors, each making a contribution to the variance of the final phenomenon, then this total variance is equal to the sum of the component variances and this makes possible the analysis of the total variance into its component factors[3]. The following sources of variance are found to be significant; between drugs, between vehicles or bases, and the drug x vehicle or base interaction. Certain factors were found to be insignificant and could therefore be ignored in the test devised for the measurement of percutaneous absorption. No difference was observed in rabbits of different sex. All rabbits chosen for test weighed between 2·5 and 3·5 kg. and no significant difference was observed for rabbits of different weights. The variation noted between individual rabbits was also found to be insignificant and in addition the difference between replicate tests was never significant.

We are indebted to Mr. J. C. Eaton, M.A., for his assistance with the statistical analysis.

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DISCUSSION

The short communication was presented by MR. J. C. MORRISON.

THE CHAIRMAN. How did the Authors arrive at the 'calculated' figure in Table II? Was the rate of absorption of the drug faster than its diffusion through the layer of ointment base on the skin?

MR. T. D. WHITTET (London). It was not justifiable to describe the absorption of materials from a 5 per cent sodium carboxymethylcellulose solution as absorption from water. Swallow and he had shown in 1942 that copper sulphate was incompatible with sodium carboxymethyl cellulose and the Authors had obtained better absorption with an incompatible material. Why was copper acetyl-acetonate chosen and was it soluble in water?

MR. S. G. E. STEVENS (London). Had any compound been used which had relatively similar solubilities in the oil and water phase? Was there any significance in the possibility of a sodium bridge carrying through the copper salts?

MR. G. R. WILKINSON (London). What was the area of skin to which the preparation was applied? How was the preparation maintained in position? Had the Authors considered that by rubbing every 15 minutes the surface layer and the subcutaneous tissue were disturbed by agitation?

DR. G. F. SOMERS (Liverpool). How was the sulphanilamide applied and what was the vehicle? Hadgraft and he had also found that in general the absorption of an oil-soluble substance was retarded if applied as an emulsion. Absorption was not always wanted.

MR. B. B. NEWBOLD (Sheffield). The differences found in the blood concentrations of sulphanilamide might not be due to biological effects, but to the insensitivity of the method used to determine the drug.

MR. MORRISON replied. The calculated figures were found by subtracting the deviation from the grand mean of all results from the average value. If there were no interaction between drug and base, the calculated and observed values should be the same. There was a possibility that the rate of absorption was faster than the rate of diffusion. A colloidal preparation of carboxymethylcellulose had been used, since water was difficult to apply to rabbits in practice, but the point was still valid. Copper acetyl-acetonate which is insoluble in water, was chosen since it could be estimated, and copper sulphate because it was astringent. They had tried a drug with approximately the same solubility in water as in oil, but they were limited by suitable assay methods. He did not support the existence of a sodium bridge. The area of application was 24 square inches: the rabbits were in boxes with their heads protruding and a barrier put down. The ointment was only rubbed on sufficiently to keep it in close contact with the skin.

THE ACTIONS OF DIGITALIS LEAF PREPARATIONS AND OF CARDIAC GLYCOSIDES ON THE ISOLATED RIGHT VENTRICLE OF THE GUINEA PIG

BY G. A. STEWART

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THIS communication is concerned with a study of the direct action of digitalis leaf preparations and of cardiac glycosides on heart muscle by employing the isolated right ventricle of the guinea pig. The muscle is housed in a specially constructed glass vessel* which permits the muscle to be oxygenated with pure oxygen without mechanical agitation. The Ringer fluid, maintained at 35°, contains a high concentration of $CaCl_2$ (2·015 g./l.) which produces a large augmentation of the amplitude of contraction. This is recorded isotonically on a kymograph by a lever system. The muscle is stimulated twice each minute from platinum electrodes connected to an electronic stimulator.

When the preparation has settled in the bath a dose of a glycoside is added. This produces an increase in amplitude which passes through a maximum and finally diminishes to zero. Simultaneously the resting length of the muscle may increase or remain unchanged, depending on the dose, during the increase to maximum amplitude and then will invariably decrease as the amplitude diminishes to zero.

Such a tracing* permits the study of the action of the glycoside on heart muscle to be undertaken in detail. The $\log_{10}$ time to/or of: first increase in amplitude, the beginning of the plateau, that is 95 per cent of the maximum amplitude, maximum amplitude, duration of plateau, maximum resting length, that is the relaxation of the muscle relative to initial length, and zero amplitude can be observed, and also the effects produced, namely rate of increase of amplitude, maximum amplitude, and maximum and minimum resting lengths, can be measured.

These metameters can be used to compare the activity of one glycoside preparation with another, as well as yielding information on the several actions which each glycoside possesses.

The study was divided into two parts. Firstly two samples of *Digitalis purpurea* leaf, of different potencies as determined by slow intravenous infusion into guinea pigs, one sample of *D. lanata* leaf, and the 3rd International Standard Preparation of *D. purpurea* were extracted at room temperature with 80 per cent (v/v) ethanol. The samples were assayed in terms of the standard on the right ventricle preparation using a 3 + 3 assay design. Only one dose was allowed to act on each isolated ventricle, and each dose was administered to six preparations. From the metameters dependent on time the results in terms of the standard ranged

* The Figures for this communication appear in the full text which is published in the December number of the Journal on pages 741 to 754.

from 33 to 42 per cent and from 65 to 74 per cent for the two samples of *D. purpurea* leaf respectively, and from 177 to 227 per cent for the sample of *D. lanata*. Within each sample none of the results was significantly different. These results are in close agreement with those obtained for the same extracts by slow intravenous infusion into intact guinea pigs namely 32 per cent and 68 per cent respectively for the two samples of *D. purpurea* and 180 per cent for the sample of *D. lanata*. The potencies from the ventricle from metameters involving changes in amplitude and resting length were higher, namely 57 to 70 per cent and 72 to 111 per cent for the two samples of *D. purpurea* respectively and 243 to 334 per cent for the sample of *D. lanata*. The limits of error are extremely wide, and the results do not therefore differ significantly from those obtained from the time metameters.

The second part of the study was concerned with the actions of digoxin, ouabain, and a sample of digitoxin assaying at 1000 I.U. per g. by the guinea pig infusion method. The experimental technique was similar to that described for the digitalis leaf preparations. The number of doses was increased to cover those from 2·5 μg. to 160 μg. inclusively in a geometric ratio of 2, which permitted a more detailed study of the influence of dose on the relationship between the various effects produced by each glycoside.

Two distinct phases were noted for doses of each glycoside on maximum amplitude. With lower doses the maximum amplitude, corrected for initial amplitude, increased as the dose increased. Beyond a certain dose, different for each glycoside, the maximum amplitude decreased with increase in dose. With lower doses the time to maximum amplitude, beginning and duration of plateau, and zero amplitude was constant while the maximum amplitude itself markedly increased. With larger doses the time to these effects became shorter with increase in dose with a concomitant reduction in maximum amplitude and an increase in the rate of increase of amplitude.

The initial increase in resting length of the muscle varied inversely with the dose for digoxin and digitoxin whereas with ouabain, up to 20 μg. the length increased with the dose. With all three glycosides doses in the middle of the range produced a decrease in the resting length with increase in dose.

The metameters dependent on time gave potency ratios in terms of digoxin of 1·24 to 3·84 for digitoxin and 0·85 to 1·52 for ouabain. Those dependent on the actual changes induced ranged from 1·58 to 2·30 for digitoxin, and for ouabain 1·55 for the decrease in resting length and 1·39 for the phase of increase of maximum amplitude with increase in dose. Other estimates were invalid.

The results for digitoxin were heterogeneous, due to the estimate of 2·24 from the metameter, time to zero amplitude. If this is excluded digitoxin has 1·66 times the activity and ouabain 1·26 times that of digoxin on the right ventricle preparation of the guinea pig.

This implies that the general therapeutic and toxic actions of the three glycosides are similar. It does not mean, however, that each glycoside

possesses only one mechanism of action. The evidence presented suggests that with low doses the glycoside is actively taken up by the muscle and used optimally at a dose which produces the greatest increase of maximum amplitude, and that larger doses may be taken up passively and more rapidly and exert their toxic action on the same mechansim controlling systolic contraction or on another mechanism which is responsive only to higher doses of glycoside. Furthermore, there must be different mechanisms controlling the change in resting length of the muscle.

The comparative results for the three glycosides obtained by slow infusion into guinea pigs were 0·51 for the sample of digitoxin and 2·03 for ouabain in terms of digoxin. These are different from those obtained on the ventricle. Digitoxin is therefore less active in the acute test *in vivo* which may reflect its greater absorption by the non-cardiac tissues such as the liver and plasma. Ouabain is twice as active as digoxin *in vivo* which suggests that it may be less readily absorbed by the extracardiac tissues.

DISCUSSION

The short communication was presented by the AUTHOR.

DR. G. B. WEST (London). Why had the right ventricle been used and not the thicker left ventricle? Had the guinea pig been chosen because it was the animal normally used for testing digitalis, and had the right ventricle in the rat or the rabbit been tried? He also enquired if similar results were obtained with normal Ringer Locke solution containing the usual amount of calcium. The addition of adrenaline might also stimulate the amplitude of contraction.

PROFESSOR J. P. TODD (Glasgow). His colleagues had attempted to record the electrical output of the heart when stimulated with digitalis; electrocardiographs were recorded, but did not demonstrate a difference between cardiotonic and cardiotoxic activities.

DR. T. E. WALLIS (London). Was there a difference between the effect of the leaf and the effect of the glycoside?

The AUTHOR, in reply, said that the right ventricle, being a thinner tissue, was more sensitive to the action of glycoside. The pharmacopoeial method used the guinea pig, and the work was being extended to other parts of that animal. Rat and rabbit hearts had been tried but were both too insensitive. If the ordinary Ringer Locke solution were used, the height of the contraction obtained was too minute for immediate observation. The addition of adrenaline had been found to produce fibrillation. Other glycosides were being screened in the hope of finding some which had the therapeutic properties of the potent glycosides, but which were less toxic. Digoxin was possibly the best of the three glycosides tested, as with an eight-fold change of dose one was still on the phase of increase of maximum amplitude, whereas it was four times for digitoxin and twice for ouabain. Beyond these doses the underlying toxic effects came in; there was a greater width of therapeutic effectiveness with digoxin. He had tried the electrocardiograph technique but there

did not seem to be any correlation between the electrical and the mechanical changes in the muscle. Both leaf and glycoside seemed to give the same pattern of effects on the heart muscle. When the leaf extract was assayed, using the ventricle preparation by the infusion method, agreement was good, but it was not good for the action of the pure glycoside. As Professor Brindle and his colleagues had shown in their infusion experiments in the guinea pig, purpurea glycoside A was more potent than digitoxin, and the difference might be due to the presence of the primary glycoside.

VEGETABLE PURGATIVES CONTAINING ANTHRACENE DERIVATIVES

PART IX. AN ALOIN-LIKE SUBSTANCE IN *Rhamnus purshiana* DC.

BY J. W. FAIRBAIRN AND V. K. MITAL

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Received June 1, 1958

A new kind of anthraquinone compound has been isolated from cascara bark (*Rhamnus purshiana*). Unlike the normal glycosides it cannot be hydrolysed by heating in acid solution, but on heating in acidified ferric chloride solution aloe-emodin and a second anthraquinone, possibly chrysophanol, are produced. Some of the physical properties are described including the ultra-violet light absorption curve which shows peaks at 268, 296 and 325 mμ. The relationship of this compound to casanthranol and to the chemical assay and biological activity of cascara is discussed.

WE have already drawn attention to the presence of an aloin-like substance in cascara bark (*Rhamnus purshiana*) and its official Extract[1] and to the paper chromatographic technique used in its detection[2]. This chromatographic method also revealed the presence of two other anthraquinone compounds and for convenience we have called the aloin-like substance, which has the highest R_F value, Compound A; the substance with the next highest R_F value, Compound B; and the third substance Compound C. In the first part of this present paper we describe the isolation and some of the properties of Compound A and later we discuss the relationship of this discovery to previous work on the active principles of cascara.

THE ISOLATION OF COMPOUND A

Several methods have been published for extracting pure glycosides from cascara and we decided to follow these methods in the first place. If pure compounds could be obtained by these means their behaviour on the chromatogram could be compared with our previous results and it was hoped that one of these pure compounds would be identical with our Compound A. Both Schindler[3] and Mühlemann[4] (who worked on the related frangula bark) acetylated a suitable extract of the bark, extracted the acetylated glycosides with benzene and fractionated on silicic acid columns. We found these methods extremely tedious and in no instance did we obtain a pure compound. Chromatographic examination of the final preparations showed that Compound A was present but was admixed with impurities. We suspect that these methods lead to considerable losses due to extensive manipulation and that the original glycosides are altered owing to prolonged exposure to adverse conditions. Sipple, King and Beal[5] used a method which did not involve acetylation; this method was also laborious and yielded a reddish brown material which was a mixture of glycosides together with various impurities. Lee and Berger[6] separated a yellow substance (casanthranol) by fractional

precipitation of a methanolic extract of cascara with acetone; they further purified the precipitate by crystallising from hot *iso*propanol. We found their method a considerable improvement on the others but the final yellow substance obtained, though rich in Compound A, was still contaminated with another anthraquinone compound together with several impurities. However, as this method was the most successful we had tried we decided to use it as a basis for further work.

Since the yellow mixture just referred to could be separated into its components by paper chromatography it seemed obvious that some form of countercurrent technique or of column chromatography on cellulose would effect separation on a larger scale. Several attempts were made to do this, using simple apparatus, but as more efficient facilities were not available we decided to fall back on the older method of fractionation by selective solvents. This decision was also influenced by the discovery we made during the preliminary work that when the yellow substance was distributed in the system nitromethane/water, most of the impurities passed into the nitromethane, while Compound A remained in the aqueous phase. However, before proceeding to this next stage, we decided to investigate some of the physical properties of Compound A by isolating a few mg. by paper chromatography and examining them. This work showed that Compound A was stable in water at 60–70° for several hours, so that it was possible to extract an aqueous solution with nitromethane in a continuous liquid/liquid extractor without decomposing the compound. We decided nevertheless to minimise the time of exposure of the aqueous phase to high temperature in the liquid/liquid extractor, by preliminary extraction with chloroform and by the use of partial vacuum.

Method Used for the Isolation of Compound A

About 1 kilo of cascara bark in No. 20 powder, was extracted to exhaustion with chloroform, to remove free anthraquinones, pigments, fats, etc. The exhausted marc was oven-dried at 70° and then percolated with anhydrous methanol under anhydrous conditions until the percolate was a pale straw colour. The percolate was evaporated to dryness under reduced pressure and the solid residue dried in a vacuum desiccator. It was then extracted with about 500 ml. of boiling *iso*propanol and the hot solution immediately filtered on a Buchner filter. On cooling the filtrate a bulky brownish yellow precipitate formed. By making several extractions with similar quantities of *iso*propanol, about 30 g. of precipitate was obtained.

The precipitate was dissolved in water (10 volumes) and transferred to a suitable liquid/liquid extractor and extracted with chloroform till the liquid syphoning over was colourless. The chloroform was replaced with nitromethane and extraction continued under reduced pressure, until the nitromethane syphoning over was colourless; this process took about 16–20 hours. After this, extraction was continued without the use of vacuum. At this higher temperature further quantities of yellow impurities were removed from the aqueous phase. Extraction was again continued until the nitromethane syphoning over was colourless. The

aqueous layer was evaporated to dryness under reduced pressure and the solid residue stored in a vacuum desiccator to remove traces of moisture.

The dried powder was extracted with several lots of boiling *iso*propanol; on cooling a bright yellow precipitate was formed. This was refluxed with dry acetone for about 15 minutes to remove traces of impurity which imparted a yellow turbidity to the acetone. The process was repeated with further volumes of acetone till the latter was colourless and chromatographic examination of the yellow precipitate showed only one spot corresponding to Compound A. The precipitate was separated and dried over P_2O_5. The yield was about 8 g. Microscopical examination showed that the substance was a fine granular powder and though many attempts were made to obtain crystals, using various solvents, we have not so far succeeded in doing so. The ultra-violet spectrum of this batch was qualitatively identical with others made by slightly different processes and also with two quantities of about 5 mg. which had been prepared by band chromatography, using the paper chromatographic method previously described[2], but the extinction values at the peaks varied quantitatively. This variation would be consistent with the presence of traces of "inert" impurities such as solvent of crystallisation or material whose ultra-violet spectrum is widely removed from that of Compound A. It is unlikely that such impurities would be anthraquinone compounds. The properties described in the following section are those common to all batches so far isolated.

Properties of Compound A

General

Compound A is a yellow to buff coloured powder; it has a sweet taste with a slight background of bitterness. In the pure form it is not very hygroscopic but if impurity is present, the substance is very hygroscopic, the resulting wet mass becoming organge-brown on continued exposure to air. It is very soluble in water, methanol and ethanol but is almost insoluble in acetone, chloroform, carbon tetrachloride and dry ether.

Melting Point and Ultra-violet Light Curve

The melting point was taken on a Kofler block and the behaviour of the substance observed microscopically under crossed nicols. At 110–120° the amorphous powder swelled and at about 150° it became brightly crystalline; these crystals melted at 154–157°. The ultra-violet light curve showed peaks at 266–268, 296 and 325 mμ.

Effect of Hydrolysing Conditions

The compound is stable in warm water (60–70°) for periods up to 10 hours. When heated in a boiling water bath with hydrochloric acid a very small quantity of free anthraquinones is produced. We have used strengths ranging from 0·1N to 3·5N and time of heating 15–30 minutes and though there is evidence that the yield of anthraquinone increases with increasing time of boiling, the yields are always low. But, there is evidence that the molecule has been effected, even by mild acid conditions, and this fact will be discussed in a subsequent paper.

Oxidation with Ferric Chloride

A few mg. of the compound were heated with 15 ml. of 3N HCl containing 25 per cent $FeCl_3$ in a boiling water bath for 15 minutes. The solution was cooled, extracted with carbon tetrachloride and the latter examined for the presence of anthraquinones by the following three methods. (*a*) On shaking a portion with NaOH solution a bright rose pink colour resulted, indicating the presence of anthraquinones: the amount present was a great deal more than that obtained when a similar quantity of Compound A was hydrolysed with acid alone as already described. (*b*) Another portion of the carbon tetrachloride extract was chromatographed by the method already described[2]: two spots corresponding to aloe-emodin and to chrysophanol were obtained. The spot corresponding to chrysophanol ($R_F = 0{\cdot}93$) was eluted and paper chromatographed, along with authentic chrysophanol, using the system devised by Hillis[7]. An R_F value of 0·77 was obtained (authentic chrysophanol 0·76). (*c*) The bulk of the carbon tetrachloride solution was concentrated and allowed to cool; crystals appeared and were shown to be aloe-emodin by position on the chromatogram[2], melting point 223°, and ultra-violet light spectrum which was identical with that of authentic aloe-emodin. The second anthraquinone present was much more soluble in carbon tetrachloride and it was difficult to get suitable crystals; the ultra-violet light spectrum of those obtained indicated that impurities were present. The melting point was 184° which is consistent with that of chrysophanol (m.p. 197°) containing traces of impurity.

We therefore conclude that ferric chloride treatment produces aloe-emodin and a second anthraquinone compound which may be chrysophanol.

Discussion

We have already pointed out[1] the similarities to and differences from *aloin* exhibited by Compound A and further work on this subject is being done. The relationship to *casanthranol*, isolated from cascara bark by Lee and Berger[6], has also been referred to[1]. Lee and Berger claim it is a glycoside of aloe-emodin anthranol and glucose to which is attached a methyltetrahydroxypentonic acid lactone and an hexitol group. Its molecular weight, empirical formula and optical rotation are given but it has no definite melting point. The stated properties differ from those of Compound A because the latter is stable in water, is not a true glycoside and contains chrysophanol (or a similar anthraquinone) as well as aloe-emodin. We have prepared several batches of casanthranol from cascara bark by following the published instructions, and have also prepared some from commercial casanthranol by re-crystallisation from *iso*-propanol. The physical properties of these batches and even the analysis for elements, and optical rotation corresponded to those published by Lee and Berger but in all instances they were shown to be a mixture of Compound A with a little Compound C and several other impurities. It is possible that the differences from Compound A are partly explained by the presence of the glycoside, Compound C, as an impurity in

casanthranol. We conclude that casanthranol is not a pure substance but is a purified fraction containing a large proportion of Compound A.

The presence of Compound A may also explain some anomalous results obtained in the *chemical* assay of cascara extracts. Mitchell[8] and Fairbairn[9] both claim they can obtain much higher results for the amount of combined anthraquinones in cascara extract than can be obtained by the method of Fairbairn and Mahran[10]. These higher results were obtained by the use of mild oxidising conditions (Mitchell, private communication) and it now seems obvious that the increased yields of anthraquinones is due to the oxidative breakdown of Compound A.

Finally the question of the relationship between Compound A and the *pharmacological activity* of cascara is important. We have not yet been able to devise a satisfactory method of chemical assay for Compound A but a preliminary experiment based on our paper chromatographic method[2] suggests that the amount present in the bark is of the order of 5 per cent. This indicates that Compound A is the major anthraquinone compound of cascara and we hope to investigate its pharmacological activity in order to establish whether the activity of the drug is mainly due to this substance.

Acknowledgements. We would like to express our sincere thanks to Westminster Laboratories Ltd. (London) for generously providing a maintenance grant to one of us (V. K. M.) and to S. B. Penick and Co. (New York) for a supply of commercial casanthranol.

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DISCUSSION

The paper was presented by DR. J. W. FAIRBAIRN.

THE CHAIRMAN. No elementary analyses were given for compound A, and the fact that it gave only a single spot on a paper chromatogram was submitted as evidence of chemical purity. Had the chromatographic work been done in more than one solvent system? It was stated that the compound was not a glycoside. Had the mixture obtained by hydrolysis with hydrochloric acid and ferric chloride been examined for the presence of sugars?

DR. W. MITCHELL (London). Lee and Berger had described casanthranol as a complex glycoside. He had found that casanthranol was not split by simple acid hydrolysis. He did not believe that ferric chloride

acted as an oxidising agent but thought that the reaction was fairly specific to iron, whether in the ferrous or the ferric state. Other oxidising agents seemed to have little or no effect, nor did nickel and cobalt.

MR. J. H. OAKLEY (London). Had the author any information on the stability of compound A in mild alkaline conditions? He had in mind the use of magnesium oxide—in preparing elixir of cascara. Was there any difference in the yield of compound A from old and new bark?

DR. T. E. WALLIS (London). It was stated that the amorphous powder swelled and became brightly crystalline. What did this mean? Did the whole become crystalline or were crystals embedded in the amorphous matrix? Did the crystals separate?

MR. C. A. JOHNSON (Nottingham). Was any information available on the pharmacological activity of the new compound and was it likely to be the main contribution towards the total activity of cascara?

MR. J. H. OAKLEY (London). Did the information about the melting point give a clue to a method for obtaining crystalline material?

DR. FAIRBAIRN replied. He hoped to co-operate with an organic chemist in elucidating the structure of compound A. They did not claim that the material was 100 per cent pure, and they realised that one spot on a chromatogram was not a good criterion of purity. They had used two systems; that described, and one using *iso*butanol-acetic acid in water, and and in both there was only one spot. As compound A was not easily split in acid conditions, he assumed it was not a true glycoside. He had tested the hydrolysate and found sugars present. They had not yet studied the stability of the compound in detail, but they had so far avoided alkaline conditions. They had used old bark. Loss of the griping effect was asserted to take place after 5 months, and no bark arrived in this country until after that time. They had used a sample of fresh bark grown here to compare the constituents with those in the commercial drug. Compound A had been observed under the microscope under crossed nicols. On warming, swelling took place and bubbles of gas were seen. Well-defined crystals then began to form which were readily visible; they melted at 155°. The activity of cascara was due to two types of glycoside—labile glycosides, which were measured by the older method, and the new compound A. His earlier method measured the labile glycosides, and corresponded well with the biological assay on mice, but appeared to represent only one-fifth of the total chemical content. Aloin had little effect on mice, and it might be that compound A also had very little effect. A quantity of compound A had been heated in a vacuum oven, and above 100° the whole mass swelled and began to decrepitate, which seemed to indicate decomposition. It did not seem a safe way of crystallising to heat to that temperature.

THE ISOLATION AND COMPARISON OF PYROGENIC FACTORS FROM *PROTEUS VULGARIS*

By J. P. Todd, J. A. M. Shaw, J. A. Blain and W. Boyle

From The School of Pharmacy, The Royal College of Science and Technology, George Street, Glasgow

Received June 1, 1958

Recent reviews on bacterial pyrogens have stressed their interest as therapeutic agents[1] and discussed their relationship to endotoxins[2].

Lipopolysaccharide may be derived both from the cells of a smooth strain of *Proteus vulgaris* and from the culture medium in which it has been grown for four days. For convenience and brevity we describe them as LPS(S) and LPS(E) respectively, the (S) referring tc the lipopolysaccharides from the cell and (E) indicating the external source. Although workers using various organisms have studied the properties of the (S) type[3,4] and others the properties of the (E) type[5], the simultaneous isolation and comparison of the relationship between the two types of pyrogenic lipopolysaccharides from any organism does not appear to have been made, and in fact the lipopolysaccharide from *Proteus vulgaris* seems to have escaped detailed study.

Proteus vulgaris grows well in inorganic and organic fluid medium. When it had been established that the LPS(S) isolated by Westphal's process[6] from cells grown in inorganic medium was similar to that obtained from cells grown in nutrient broth, and that the latter yielded heavier growth of cells, the LPS(S) was more conveniently obtained in quantity from the cells grown in the organic medium. The yield of LPS(S) from the dried cells (5–10 per cent) contained 40–50 per cent of nucleic acid which could not be reduced below 2 per cent. (Table I.) As the loss during purification by this method is considerable five sedimentations were not exceeded in subsequent purifications.

TABLE I

Removal of nucleic acid from LPS(S) by washing and sedimentation at 105,000 *g*

Sedimentation	1	2	3	4	5
Per cent residual Nucleic acid ..	15	7	5	2–3	2–3

In all instances the LPS(E) was obtained from the cell-free filtrate from the same organism, grown in inorganic medium for four days, by preliminary evaporation to low volume under reduced pressure followed by removal of inorganic salts by dialysis, extraction of bacterial protein with phenol, alcohol precipitation and repeated sedimentation in a high-speed centrifuge at values of up to 105,000 *g*, followed by freeze-drying.

The LPS(E) after concentration of the fluid medium and then dialysing was found to contain an antigenic protein-lipopolysaccharide complex and was completely free from nucleic acid which made purification of this

fraction easier. The protein LPS(E) complex could either be obtained as such by evaporation to suitable bulk and sedimentation in the high-speed centrifuge: or the protein could be removed by extraction with phenol as in the Westphal method for extraction from the dried cell.

Comparison of the two lipopolysaccharides showed that both contained a chloroform-soluble lipid, which could be split off on acid hydrolysis. Absorption spectra in the Dische test[7,8], showed a similar pattern: peaks at 408 mμ and 505 mμ indicated similar ratios of hexose to heptose in both compounds. Both fractions were shown by paper chromatography to contain hexosamine. On analysis the preliminary figures shown in Table II were obtained. Some further evidence of purity and homogeneity of the

TABLE II

COMPARISON OF LPS(S) AND LPS(E)

	C	H	N	P	Reducing sugars as glucose	Lipid	Minimum pyrogenic dose
LPS(S)	41·21	7·28	2·28	1·95	34 per cent	30–32 per cent	0·005 μg./kg.
LPS(E)	43·07	7·86	2·10	1·72	30 per cent	32–34 per cent	0·005 μg./kg.

two lipopolysaccharides was sought and both products were tested by the agar diffusion precipitin test[9], using the lipopolysaccharide as "antigen" against the serum of rabbits previously immunised against *Proteus vulgaris* cell suspension. The LPS(S) and LPS(E) in the prepurification stages gave two and several lines respectively. After sedimentation at 105,000 *g*, the LPS(S) gave only one zone while the LPS(E) gave a major coincident zone and a very faint zone suggesting an impurity. A mixture of the two lipopolysaccharides gave one major zone along with the very faint zone already mentioned. This was taken to indicate a close relationship but further detailed examination is in progress.

Acknowledgements. This research programme has been supported by a grant from the Scottish Hospital Endowments Research Trust. One of us (W. B.) thanks The Pharmaceutical Society of Great Britain and the Cross Trust for provision of scholarships during the tenure of which this work was carried out. The dried cells were produced for us at the Antibiotics Research Station, (M. R. C.), by the kindness and courtesy of Dr. Kelly. We would like to acknowledge technical assistance from Mr. David Rountree and Miss Rita Laird.

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DISCUSSION

The short communication was presented by MR. W. BOYLE.

THE CHAIRMAN. In what form was the phosphorus present in L.P.S.(S) and L.P.S.(E): from Table II there seemed to be little difference in their phosphorus contents. It seemed reasonable to suppose that the lipopolysaccharide required some portion of its molecule to be split off before part of it could pass through the bacterial cell into the culture medium. Might L.P.S.(S) be similarly split during its process of purification in view of the composition being so similar to that of L.P.S.(E)?

DR. J. G. DARE (Leeds). He had grown the *Proteus vulgaris* on surface cultures on solid media, washed them off carefully, to give a very concentrated suspension which, after being incubated, was filtered. When the filtrate was poured into another solvent, such as acetone, a precipitate of very highly active pyrogenic material was obtained in a yield corresponding to about 1 per cent in the original filtrate. By this method he avoided the lengthy and tedious concentration under reduced pressure. It was possible to obtain preparations which were active in rabbits in doses of less than 0·01μg./kg. He had tried a number of purification processes such as those recommended by Morgan, and he had always got back almost a quantitative yield (about 80 per cent) of the original material. He did not know whether that constituted purification or some loss in the process. Had the authors any reasons for thinking that his method might be unsatisfactory?

MR. G. A. STEWART (Dartford). It was stated that there was a hypothermic reaction with one preparation. Did they consider this to be a dual action of the pyrogen or due to some impurity associated with it?

MR. T. D. WHITTET (London). Had time-temperature curves for the two substances been compared?

DR. L. SAUNDERS (London). Might not these materials be break-down products from the cell wall which was being continually renewed? Had the ultra-violet absorption spectrum of the lipopolysaccharide been examined, and if so did it show unsaturated fatty acid in the active portion?

MR. H. D. C. RAPSON (Betchworth). Could an analogy be drawn between allergic reactions, such as from pollen extracts, and the pyrogen reaction?

MR. T. D. WHITTET (London). A large number of mucopolysaccharides had been extracted from allergic extracts recently. He had had some, and had not found any to be pyrogenic.

MR. BOYLE replied. The phosphorus figures quoted were for total phosphorus, and they had no evidence how it occurred. In the examination of the crude E material the nucleic acid absorption was at 260mμ, with much masking, probably due to the protein. After phenol extraction the polysaccharide material showed no peak at 260 mμ. It might be that in this extraction the nucleic acid had gone into the phenol layer and the polysaccharide into the aqueous layer. The surface culture method gave a great deal of extraneous matter, which they thought

would make purification more difficult. The material obtained from the cell-free filtrate had been used for some 10 years, and in that time very few toxic reactions had occurred in its use in rabbits. One of their aims was that eventually the materials would be used for clinical trials. They had been looking for an agent with as low toxicity as possible, and this method seemed to offer it. He agreed that the order of activity was not an indication of purity. After injection of these materials the first reaction was often hypothermic, and hyperthermia would occur later. Doses of about 10 μg. resulted in a temperature rise of 2-3° which might persists for 6 hours. Then it began to fall. If the same dose was again injected, there was a dramatic fall of temperature of about the same amount. They had found the curves to be almost exactly superimposable. With the E lipopolysaccharide the latent period was slightly shorter. When they gave higher doses, of about 1 μg., there was a double peak response, but curves were still superimposable. They had not examined the ultra-violet absorption of the lipopolysaccharide.

PROFESSOR TODD replied. To obtain the bacterial lipopolysaccharide by evaporating the medium was indeed a laborious method, but they had been anxious to find the substance which was present in injections. He agreed the indications were that the same substance was obtained by both methods. He thought that the presence of nucleic acid in one and its absence from the other was due to the location of the lipopolysaccharides in bacterial cell walls. The phenolic extraction disintegrated the cell and one got nucleic acid with it. There were two aspects to the fall in temperature. At the beginning of some injections there was a fall and then a continued rise, but with considerable overdosages there was a fall and no rise at all, the temperature remaining below the base line, presumably approaching the toxic level.

THE SURFACE ACTIVITIES OF α-AND β-(ACYL) LYSOLECITHINS

By N. Robinson and L. Saunders

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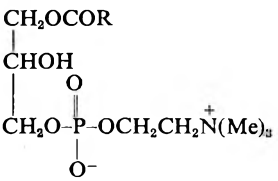
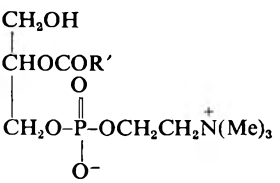
Received June 1, 1958

The lysolecithins can be regarded as derivatives of monoglycerides and would be expected to possess α or β acyl linkages; the two lysolecithins examined in the present work are therefore considered to have the structures:—



α-(acyl) lysolecithin



β-(acyl) lysolecithin

R and R′ are unsaturated and saturated hydrocarbon chains respectively

The sample of α-lysolecithin, obtained by acetic acid treatment of the choline plasmalogen of ox heart[1], possessed a high degree of unsaturation, whereas β-lysolecithin, obtained by the action of snake venom on lecithin isolated from egg yolk, contained a fatty acid moiety which had a very low degree of unsaturation.

The present investigation was undertaken to compare the surface activity of the two lysolecithins. Each compound possesses two distinctly different regions in the molecule, a fatty acid chain of non-polar character and another region containing the phosphate-choline group of a polar nature. The distance between these two regions in the α-lysolecithin is greater than in the β-lysolecithin and hence the amphipathic character was expected to be more pronounced in the former. On the other hand, the presence of unsaturated linkages in the fatty acid radical of the α compound gives the molecule an increased affinity for water which is likely to lessen its surface active properties.

Preparation of lysolecithins. β-(acyl) lysolecithin was prepared by treating lecithin obtained from egg yolks with Russell viper venom according to the method previously reported[2]. The α-(acyl) lysolecithin was a gift from Dr. G. M. Gray, Lister Institute of Preventive Medicine. Analysis of the α-compound (made by the Lister Institute) and the β-compound (the authors') gave the following:—

	α	β
Nitrogen (per cent on dry wt.)	2·76	2·72
Phosphorus (per cent on dry wt.)	5·9	5·98
N:P ratio	1:0·97	1:1·02
Double bonds/mole fatty acid ester	2·22	0·12

The mean molecular weights calculated from the nitrogen and phosphorus contents were 518 and 516 respectively.

Preparation of aqueous sols. Sols of each lysolecithin were prepared by evaporating a sample of the stock solution of the compound to dryness, dissolving the known weight of material in water which had been previously distilled, passed down a small column of mixed strong ion exchange resins and then saturated with nitrogen. The sols were made up to volume and diluted when required; the α-lysolecithin sol had a pale yellow colour and the β-lysolecithin sol was colourless; both were optically clear.

Surface tension apparatus. The surface tension measurements were made at 25° and 40° using the ring (dynamic[3]) and Wilhelmy plate (static[4]) methods employing a chainomatic balance[5]. The Wilhelmy plate method was used for examining very dilute solutions to obtain surface areas.

Results and discussion. The effects of α- and β-lysolecithins on the surface tension of water are shown in Figure 1. At 25° and concentrations of 0·1 per cent w/v the α-compound lowered the surface tension of water to 39·0 dyne/cm., whereas the β-compound only lowered the surface tension to 41·0 dyne/cm. At 40° the α-compound again showed the greater surface activity by lowering the surface tension to 37·2 dyne/cm. compared with a lowering to 38·7 dyne/cm. obtained with the β-compound. The

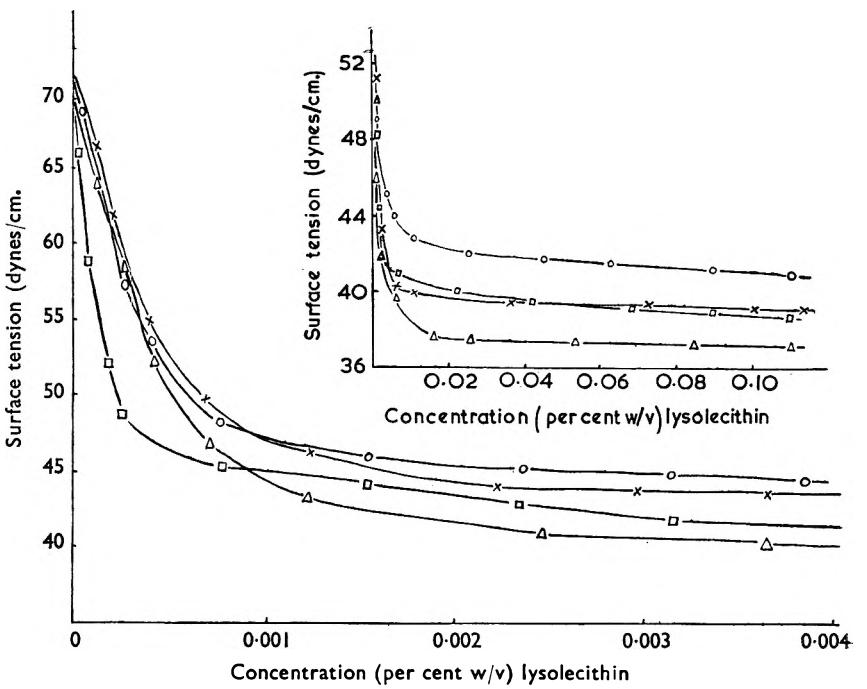


FIG. 1. Effect of α- and β-(acyl)lysolecithins on the surface tension of water. ×, α-Lysolecithin at 25°; ○, β-lysolecithin at 25°; △, α-lysolecithin at 40°; □, β-lysolecithin at 40°.

tendency for the α-compound to show the greater effect was found throughout the range of concentrations examined except in the dilute region.

Below a concentration of 0·001 per cent w/v, at both temperatures the β-lysolecithin lowered the surface tension of water more than α-lysolecithin. It is probable that below this concentration the solutes were present as single molecules or small aggregates. In this case the greater surface activity of the β-compound can be attributed to the saturated fatty acid moiety which gives a more pronounced distinction between the lipophilic and hydrophilic regions. It is suggested that this increased amphipathic character assists molecules of the β-lysolecithin to form micelles more readily than the α-lysolecithin, so that above 0·001 per cent w/v fewer single molecules are present in equilibrium with micelles. This results in a smaller lowering of the surface tension of water with β-lysolecithin in the higher concentration range compared with that of the α-compound.

We have suggested elsewhere[5] that the marked change in the lowering of the surface tension of water within the concentration range 1 to 2×10^{-3} per cent w/v by β-(acyl) lysolecithin at 20° is due to the formation of large micelles. The surface tension/concentration curves in Figure 1 indicate that a critical micelle concentration exists also for the α-compound in this region.

In the dilute region the areas of the α- and β-lysolecithins at the interface were calculated to be 101 and 90 Å^2 per molecule at zero surface pressure ($\gamma_{water}-\gamma_{sol}$) decreasing to 57 and 66 Å^2 per molecule respectively at a surface pressure of 12·5 dyne/cm. at 40°. These values obtained by using the approximate adsorption isotherm show that on increasing the surface pressure the area of the unsaturated α-compound is considerably reduced. This comparison between the two lysolecithins indicates that considerable solution of the α-compound is probably taking place away from the surface film.

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DISCUSSION

This short communication was presented by MR. N. ROBINSON.

THE CHAIRMAN. What was the evidence that the acyl groups were in the α or β positions? Had the preparations been examined by polarised light? It was well known that the acyl group could wander from the β to the α position. Had the possibility been considered?

MR. A. R. ROGERS (Brighton). Was there any information available whether the two double bonds in the α-compound were conjugated or not? He suggested it might be possible to hydrogenate this unsaturated compound if the double bonds were conjugated without destruction of the

rest of the molecule to give a closer comparison of what happened to the fatty acid group in the α and β positions.

MR. H. D. C. RAPSON (Betchworth). It would be interesting to examine the compounds in the semi-solid state. Had the possibility of electro-chemical reduction been considered?

DR. G. B. WEST (London). He noticed that the α-lysolecithin was derived from mammalian sources whereas the β-compound was derived from avian sources. Was this a peculiarity of the type of species or did both occur in the ox and the egg?

MR. ROBINSON replied. By oxidation and examination of the resulting products Hannahan, Long and Penny had established the position of the β-group in 1954. The α-compound had been given to them, and its constitution had been proved in the work referred to in the first reference which was to be published. He agreed that migration favoured the α position. He could give no information about conjugation. They had not been able to analyse the α-compound because they had been given less than 100 mg. Their work on the α-compound had been limited to the surface on account of the small amount available. He had tried electro-chemical reduction with lecithin, but he had not had much success.

DR. SAUNDERS replied. In preliminary experiments under crossed nicols no streaming or birefringence had been observed. With the solid preparations under crossed nicols they had reported a crystalline appearance, and he had no doubt that with better technique they would be able to demonstrate streaming. It must be present in the very viscous sols. The β-compound, which they made, was not extracted directly from natural sources. The lecithin was first made from eggs, and then treated with an enzyme.

A NOTE ON THE PHARMACOLOGY OF RESCINNAMINE AND SERPENTINE

By M. S. Zoha*, S. M. Kirpekar† and J. J. Lewis

From the Department of Materia Medica and Therapeutics
University of Glasgow

Received April 29, 1958

The pharmacological properties of rescinnamine and serpentine have been studied. Both lower the blood pressure of anaesthetised cats but rescinnamine has a prolonged effect whilst serpentine has only a brief action. Rescinnamine does not antagonise the effects of adrenaline or noradrenaline on the blood pressure of the cat or on the isolated heart, auricles or intestine. Serpentine antagonises vasoconstriction due to adrenaline and noradrenaline in the isolated perfused rat hindquarters but not that due to 5-hydroxytryptamine creatine sulphate, barium chloride or pitressin. Serpentine reversibly antagonises the pressor responses to bilateral carotid occlusion, central vagal stimulation and splanchnic stimulation. Rescinnamine irreversibly antagonises these responses and also the responses from compression of the abdominal aorta and hypoxia, which suggests both alkaloids have an action upon the sympathetic nervous system. Rescinnamine also has some direct depressant effects on cardiac, intestinal and skeletal muscle.

Clinical trials of rescinnamine by Smirk and McQueen[1] and by Hershberger, Dennis and Moyer[2] have indicated that there is apparently no important difference between the hypotensive effects of rescinnamine and those of reserpine. It was observed that mental symptoms occurring in patients treated with reserpine were often relieved by changing to rescinnamine without any adverse influence upon the control of the blood pressure. Rescinnamine and serpentine have been reported to have hypotensive properties in animals[3–5], but rescinnamine displays no antagonism to the pressor effects of adrenaline or noradrenaline on the blood pressure of the cat, instead there is a slight enhancement of the pressor response[6]. Rescinnamine inhibits the pressor responses to bilateral carotid occlusion, electrical stimulation of the central end of the cut vagus and the pressor response to hypoxia. On the other hand serpentine does not antagonise the pressor effects of adrenaline or noradrenaline and does not inhibit the response of the isolated guinea pig seminal vesicles to adrenaline[5]. Rises in the blood pressure of the cat following electrical stimulation of the cut central ends of the sciatic nerve or vagus, or following stimulation of the splanchnic nerve, or occlusion of the carotid arteries are either not inhibited, or only inhibited to a minor degree, by serpentine[7].

The information available about the effects of rescinnamine or serpentine on the cat or on isolated tissues and organs, is limited and since both alkaloids are present in some rauwolfia preparations which are used clinically we felt it worthwhile to amplify some of the previous studies.

* Pakistan Government Scholar.
† Squibb Foundation Fellow.

Materials and Methods

Perfusion fluids. The composition of these has been described previously[8].

Drugs were dissolved in the appropriate saline solution before use. Rescinnamine was dissolved in dilute acetic acid.

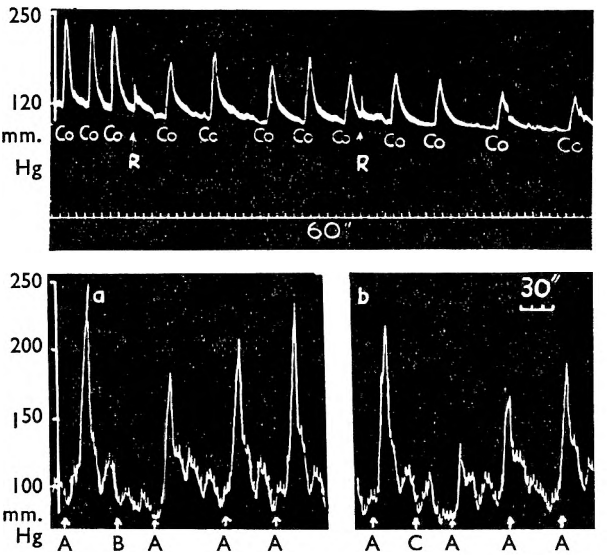

FIG. 1. Influence of rescinnamine and serpentine on the pressor response to bilateral carotid occlusion.

Upper Tracing. Cat, chloralose anaesthesia. Blood pressure record taken from the femoral artery. Drugs administered intravenously. At Co, bilateral carotid occlusion for 30 sec. At R, rescinnamine 0·75 mg./kg.

Lower Tracing. Cat, chloralose anaesthesia. Blood pressure record taken from the femoral artery. Drugs administered intravenously. At A, bilateral carotid occlusion for 25 sec. At B, serpentine 1·0 mg./kg. At C, serpentine 2·0 mg./kg. Record (b) 10 min. after (a).

The following preparations and techniques were used.

In pentobarbitone or chloralose-anaesthetised cats constant pressor responses were obtained to: (*a*) intravenous injection of adrenaline (Ad) or noradrenaline (NA), (*b*) clamping of both common carotid arteries, (*c*) stimulation of the cut central end of the vagus, (*d*) stimulation of the cut central end of the sciatic nerve, (*e*) stimulation of the cut central end of the greater splanchnic nerve, (*f*) compression of the abdominal aorta above the level of the coeliac artery and (*g*) hypoxia by inhalation of a 95 per cent N_2, 5 per cent CO_2 mixture. Nerves were stimulated by square impulses from a Dobbie McInnes stimulator at 5 to 20 volts, frequency of 1000 to 1400 per minute, pulse width 0·5 to 3·0 msec.

A constant hypertension was maintained by intravenous infusion at 1 ml./min. of a solution containing from 50 to 100 μg./ml. of Ad or NA. Depressor responses were obtained by injection of acetylcholine (ACh) or histamine acid phosphate (Hm). Contractions of the nictitating

membrane were obtained in response to electrical stimulation of preganglionic fibres of the cervical sympathetic.

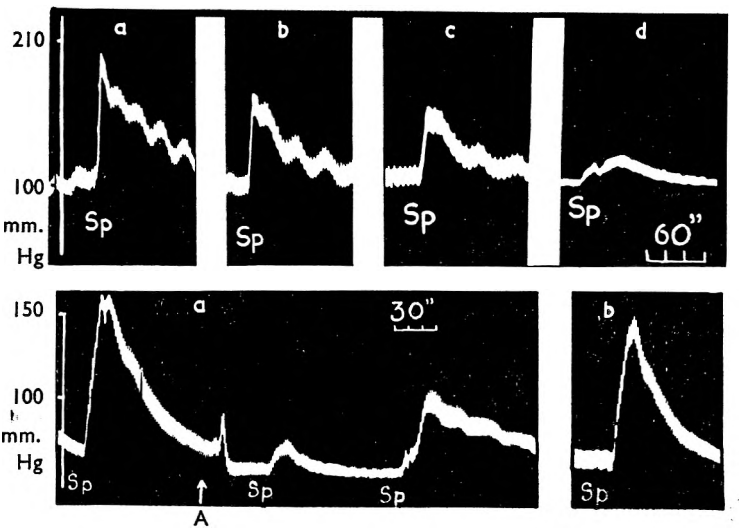

FIG. 2. Influence of rescinnamine and serpentine on the pressor response to stimulation of the splanchnic nerve.

Upper Tracing. Cat, pentobarbitone anaesthesia. Blood pressure record taken from the common carotid artery. Drugs administered intravenously.

At Sp, stimulation of the central end of the greater splanchnic nerve for 15 sec. (square impulses, 10 v., frequency 1,000/min. pulse width, 3 msec.).

(a) before rescinnamine.
(b) 10 min. after 1 mg./kg. rescinnamine.
(c) 50 " " "
(d) 180 " " "

Lower Tracing. Cat, chloralose anaesthesia. Blood pressure record taken from the common carotid artery. Drugs administered intravenously.

At Sp, stimulation of the central end of the greater splanchnic nerve for 30 sec. (square impulses 10 v., frequency 1,200/min. pulse width, 1·5 msec.).

At A, serpentine 1 mg.

Record (b), 20 min. after serpentine.

The frog rectus abdominis muscle was suspended in frog Ringer's solution at room temperature, isolated strips of rabbit duodenum were suspended in oxygenated Locke's solution at 37°, strips of guinea pig ileum in oxygenated Tyrode's solution at 30° and strips of horse carotid arteries in oxygenated Tyrode's solution at 36°. Isolated kitten hearts were perfused by Langendorff's method using oxygenated, double-glucose Locke's solution at 37° and this solution at 29° was used for isolated guinea pig auricles. The isolated rat hindquarters were perfused by oxygenated Locke's solution at room temperature using Gaddum's drop recorder to measure the out-flow.

In experiments with isolated tissues all drug concentrations, unless otherwise mentioned, refer to the final bath concentration in μg./ml. In

experiments on intact cats the doses are expressed as μg. or mg. of drug per kg.

Results. These are presented in Table I.

DISCUSSION

Rescinnamine causes an immediate but short-lived fall in the blood pressure of the anaesthetised cat but our observations do not point to

TABLE I

COMPARISON OF PHARMACOLOGICAL EFFECTS OF RESCINNAMINE AND SERPENTINE

Preparation	Rescinnamine	Serpentine
B.P. Anaesthetised cat	0·5–2 mg. Immediate sharp fall, returning to base line in 10–15 min. No significant change until 3–4 hr. later, when B.P. dropped to 50–80 mm. Hg with bradycardia.	1–2 mg. Immediate short-lived fall.
	0·5–1 mg. No or slight reduction in pressor response to Ad and NA (0·5–2 μg.)	1–2 mg. Antagonism to Ad (1–4 μg.) response more effective than to 1–4 μg. NA.
	0·5–1 mg. No modification of ACh or Hm (1–2 μg.) depressor responses.	0·5–1 mg. As rescinnamine.
	1 mg. Slight reduction in Ad or NA induced hypertension.	1–2 mg. Little or no effect on the hypertension.
	2–4 mg. Irreversibly lowered hypertension to normal in a few minutes.	
Vasopressor reflexes (i) Bilateral occlusion of common carotid arteries (see Fig. 1)	0·5–1 mg.	1–2 mg. Pressor effects in (i) reduced and reversible.
(ii) Compression of abdominal aorta (iii) Electrical stimulation of greater splanchnic nerve (see Fig. 2)	Pressor responses significantly reduced but not completely abolished. Maximum reached after 30–60 min.	(ii) No response. (iii) Reversible effect.
(iv) Electrical stimulation of central end of cut vagus (see Fig. 3) (v) Hypnoxia (vi) Electrical stimulation of central and of cut sciatic nerve	Pressor effects abolished in 15–30 min.	(iv) Effects reduced and reversible and of short duration. (v) No response. (vi) Effects reduced and reversible and of short duration.
Nictitating membrane (see Fig. 4)	1–2 mg. No direct stimulant or immediate effect on magnitude of electrically induced contraction, but after 30 min. significant reduction in amplitude with a maximum after 3 hr.	1–2 mg. No direct effect, significant reduction in electrically induced contractions. 3–4 mg. abolished response and also antagonised Ad-induced contractions.
Cardiac muscle	Perfusion of a solution containing 1 μg./ml.	10 μg./ml.
(i) Kitten heart	Irreversible decrease in rate and amplitude gradual in onset. Outflow decreased. Increased rate and amplitude produced by Ad and NA (0·5 μg.) were not altered by 25–50 μg.	Initial stimulation, then decreased in amplitude and rate. Outflow not significantly altered.
(ii) Guinea pig auricles	2–8 μg. Immediate reversible reduction in rate and amplitude. No effect on responses to Ad or NA (0·02 μg.)	1·5–10 μg. Reversible decrease in rate and amplitude. No effect on response to Ad or NA (0·02 μg.). Antagonised depressant effects of ACh 1–2 μg.).

TABLE I—*Continued*

Preparation	Rescinnamine	Serpentine
Vascular smooth muscle (i) Horse carotid artery	10 μg. Little or no direct effect.	10 μg. As rescinnamine.
	2–40 μg. Relaxed the sustained contractions induced by Ad and NA (1–2 μg.), 5 HT (10–50 μg.), ACh (0·02–0·1 μg.) and Hm (1–2 μg.).	10–25 μg. Antagonised stimulant effect of Ad and NA (0·1–2 μg.) and 50–70 μg. antagonised contractions induced by ACh (0·1–0·5 μg.).
(ii) Rat hindquarters	0·1–1 μg. Little or no vasodilatation.	10 μg. Reversible increase in outflow. Antagonism of the vasoconstrictor effects of Ad, NA (0·2–1 μg.) but not to 5-HT (0·01–1·5 μg.), $BaCl_2$ (0·2–1·0 mg.) or pitressin (0·01–0·02 units) see Fig. 5).
Intestinal smooth muscle (i) Guinea pig ileum	1·5–3 μg. In some preparations had a direct stimulant effect which was inhibited by atropine.	1–12·5 μg. No direct effect. Inhibition of contraction induced by 0·2–1·0 μg. of ACh; rapid recovery.
	2–20 μg. Antagonised ACh and Hm (0·04–0·1 μg.) induced contractions. Recovery was complete, but for Hm was prolonged. (see Fig. 6)	
(ii) Rabbit duodenum	No direct effect and no modification of ACh and Ad.	1–10 μg. Slightly stimulated, but 25–100 μg. inhibited peristaltic movement. 2·5–10 μg. antagonised ACh-induced contractions (0·02–0·2 μg.) but no effect on relaxation produced by Ad or NA (0·05–0·2 μg.).
Skeletal muscle Frog rectus	5–50 μg. Direct stimulant effect (Fig. 7). Not influenced by atropine or tubocurarine (5–10 μg.). Antagonised ACh-induced (0·1–0·2 μg.) contractions. This was more marked with prolonged contact (Fig. 7*a*) than with a few minutes contact (Fig. 7*b*).	2–10 μg. Antagonism of ACh (0·25 μg.), nicotine hydrogen tartrate (1·5–2 μg.) and C10 (1–1·5 μg.) induced contractions. Potentiates antagonism of tubocurarine (0·5–1 μg.).

rescinnamine having a definite hypotensive action in the normotensive cat. Rescinnamine does not antagonise the pressor responses to Ad or NA on the blood pressure of the cat nor does it show any marked antagonism to their characteristic effects in isolated tissue preparations. Rescinnamine antagonism to Ad, NA, 5-HT and Hm on isolated strips of artery seems to be non-specific. Gillis and Lewis[9] have shown that reserpine-antagonism to contractions of the guinea pig ileum induced by ACh, Hm, 5-HT or $BaCl_2$ is non-specific in nature and that the effects of reserpine can be antagonised to some extent by certain intermediates of the tricarboxylic acid cycle. It is possible that rescinnamine which is closely allied chemically to reserpine may act in a similar way. Our observations do not support the findings of McQueen and Blackman[10] who have shown rescinnamine to have a vasodilator effect on the isolated innervated and denervated hindquarters of the rat or rabbit.

Rescinnamine antagonises or may reverse the pressor responses to hypoxia and to stimulation of the central end of the cut vagus, whilst pressor responses after bilateral carotid artery occlusion, splanchnic nerve stimulation and compression of the abdominal aorta are considerably reduced. Rescinnamine acts after a latent period and seems to

exert its maximum effect some 30 to 60 minutes after its administration. These reflex pressor responses may have the same mechanism of action, in the liberation of Ad and NA from the endings of adrenergic nerves, the reflex being mediated through the higher centres of the brain. The fact that adrenergic and ganglion blocking agents depress these reflexes supports this view[11]. Antagonism shown to these pressor reflexes indicates that rescinnamine may interfere with sympathetic activity in the central nervous system rather than at the periphery because it shows no antagonism to the peripheral effects of injected Ad and NA. Rescinnamine also

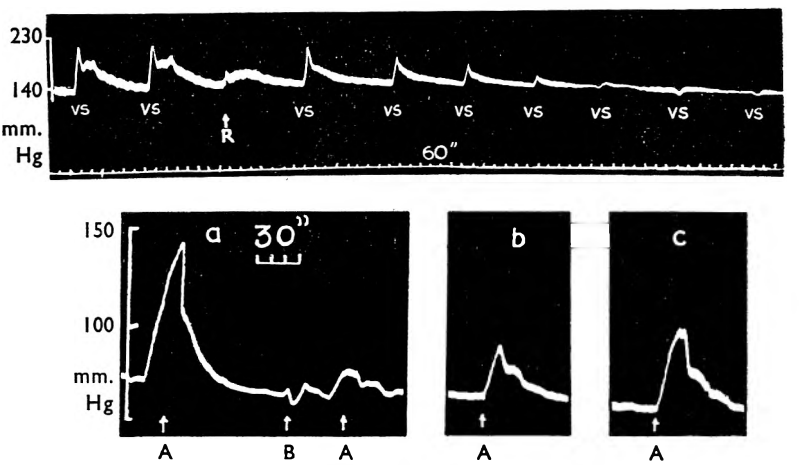

FIG. 3. Effects of rescinnamine and serpentine on the pressor response to stimulation of the cut central end of the vagus.
Upper Record. Cat, pentobarbitone anaesthesia. Blood pressure record taken from the carotid artery. Drugs administered intravenously. At Vs, stimulation of the cut central end of the vagus for 30 sec. (square impulses 15 v., frequency 1,000/min. pulse width 1 msec.). At R, rescinnamine 0·75 mg./kg.
Lower Record. Cat, chloralose anaesthesia. Blood pressure record taken from the carotid artery. Drugs administered intravenously. At A, stimulation of the cut central end of the vagus for 30 sec. (square impulses, 10 v., frequency 1,200/min. pulse width 0·75 msec.). At B, serpentine 1 mg./kg.
Records (b) and (c), 6 and 10 min. after serpentine.

considerably reduces the response of the nictitating membrane to stimulation of preganglionic sympathetic fibres which may be due to its effect on the ganglia. Rescinnamine also has some direct effects on smooth muscle cells; thus it inhibits ACh and Hm induced contractions of the guinea pig ileum and it antagonises the stimulant effects of drugs on artery strips. It also has a depressant effect upon the isolated heart and auricles and antagonises the stimulant effects of ACh on the frog rectus. Like reserpine[12], rescinnamine causes a slow contraction of the frog rectus abdominis muscle, which with reserpine, has been attributed to release of potassium ions. The character of the contraction of the rectus muscle in response to potassium ions is, however, dissimilar to that caused by rescinnamine.

Serpentine has some adrenergic blocking activity. On the blood pressure of the anaesthetised cat serpentine antagonises the response to Ad more than to NA. In higher doses it antagonises the vasoconstriction

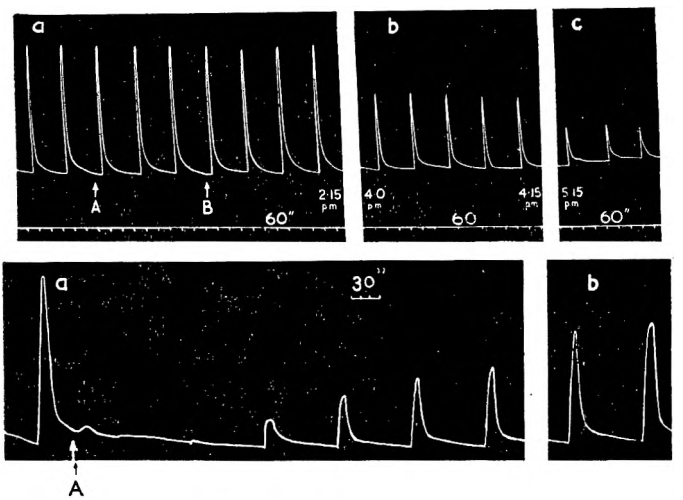

FIG. 4. Effects of rescinnamine and serpentine on the nictitating membrane.
Upper Record. Cat, pentobarbitone anaesthesia. Contractions of the nictitating membrane elicited at intervals of 3 min. by preganglionic stimulation for 30 sec. (square impulses, 10 v., frequency 1,000/min. pulse width 1 msec.). At A, rescinnamine 0·5 mg./kg. intravenously. At B, rescinnamine 2·0 mg./kg. intravenously.
Lower Record. Cat, chloralose anaesthesia. Contractions of the nictitating membrane elicited at intervals of 3 min. by preganglionic stimulation for 15 sec. (square impulses, 20 v., frequency 1,200/min. pulse width 2 msec.). At A, serpentine 2 mg./kg. Record (b), 40 min. after serpentine.

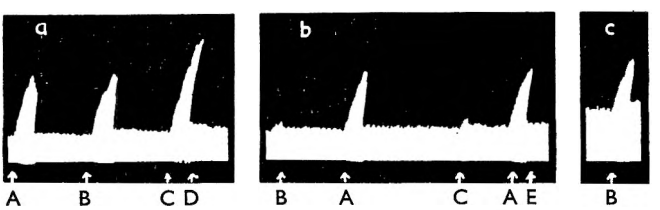

FIG. 5. Isolated rat hindquarters perfused with Locke's solution.
At A, 1·5 μg. of 5-HT. B, 0·01 μg. of Ad. C, 0·015 μg. of Ad. D, perfusion with 10 μg./ml. of serpentine. E, perfusion with Locke's solution.
Record (b), 20 min. after serpentine perfusion.
Record (c), 20 min. after perfusion with Locke's solution.

produced both by Ad and NA on isolated perfused rat hindquarters but it is ineffective against vasoconstriction caused by 5-HT, $BaCl_2$ or pitressin. It also antagonises Ad and NA induced contractions of strips of carotid

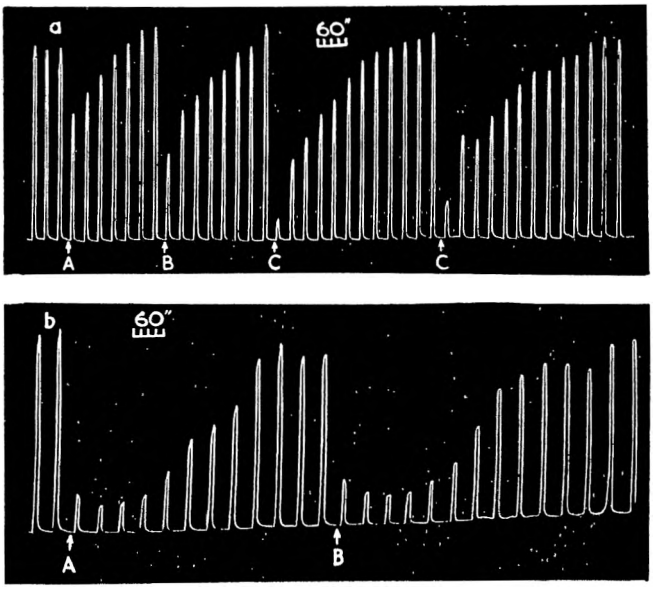

FIG. 6. The effects of rescinnamine on ACh and Hm induced contractions of the guinea pig ileum.

(a) All contractions produced by ACh (0·08 μg./ml.)
At A, rescinnamine 5 μg./ml.
At B, ,, 10 ,,
At C, ,, 20 ,,

(b) All contractions produced by Hm (0·1 μg./ml.).
At A, rescinnamine 8 μg./ml.
At B, ,, 4 ,,

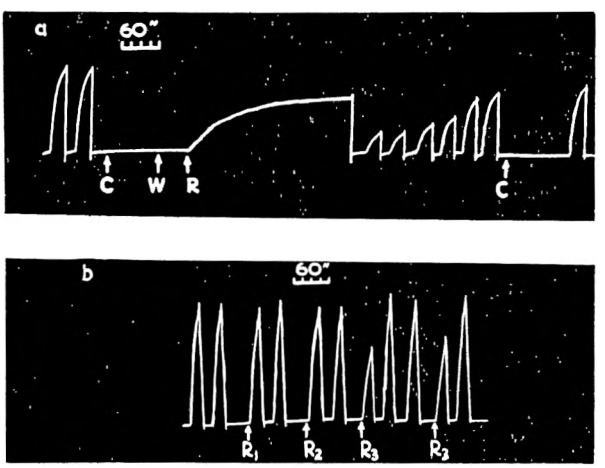

FIG. 7. The effects of rescinnamine on the isolated frog rectus abdominis muscle.

(a) Unlabelled contractions produced by ACh (0·15 μg./ml.)
At C, control solution. At W, wash out. At R, rescinnamine 20 μg./ml. for 20 min.

(b) All contractions produced by ACh (0·10 μg./ml.)
At R_1, rescinnamine 10 μg./ml. for 1 min.
At R_2, ,, 20 ,, ,,
At R_3, ,, 50 ,, ,,

arteries. Serpentine antagonises the vasopressor reflexes due to bilateral carotid occlusion and stimulation of the cut central ends of the vagus, sciatic and splanchnic nerves. Our results differ from those of Bein and Gross[7] who have reported that serpentine does not block these reflexes.

Apart from an initial short-lived fall in blood pressure, rescinnamine acts after a latent period but serpentine acts immediately and its effects wear off completely and rapidly. Serpentine antagonism is reversible but that of rescinnamine is irreversible.

Acknowledgements. We are indebted to Dr. C. D. Falconer of Ciba Laboratories Ltd., and Mr. J. A. Lumley of Riker Laboratories Ltd., for supplies of rescinnamine and serpentine.

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DISCUSSION

The paper was presented by MR. S. M. KIRPEKAR.

THE CHAIRMAN. In *Rauwolfia serpentina* reserpine was the most important alkaloid, but a great many other alkaloids were also present, including rescinnamine and serpentine. Had rescinnamine or serpentine any properties which suggested that they might replace reserpine in clinical use. Had the Authors satisfied themselves of the purity of their specimens?

DR. G. F. SOMERS (Liverpool). An important aspect of the paper was the differing results from those obtained by Bein and Gross.

MR. C. A. JOHNSON (Nottingham). The Chairman had suggested that reserpine was the main alkaloid of rauwolfia. The other alkaloids were present in much greater proportion, and the presence of reserpine was only recognised many years after such alkaloids as ajmaline and serpentine had been isolated. Did the results obtained on rescinnamine support the contention of clinicians that the hypotensive action of reserpine and rescinnamine were not significantly different, either quantitatively or qualitatively? Both of those alkaloids were closely similar in chemical properties, in contradistinction to serpentine, and both could be hydrolysed to methyl reserpate and a carboxylic acid. Had methyl reserpate an activity similar to that of rescinnamine or reserpine, or was the hypotensive effect reduced by such a hydrolysis? He believed the B.P.C. assay for

total alkaloids of rauwolfia did not extract and determine reserpine. It was the total alkaloids, less these two weakly basic alkaloids, reserpine and rescinnamine, which were determined.

DR. J. W. FAIRBAIRN (London). Was there a quantitative difference in effect between methyl reserpate and reserpine in animals?

DR. W. MITCHELL (London). It seemed surprising if the activity of methyl reserpate had not been determined.

MR. KIRPEKAR replied. Rescinnamine resembled reserpine but serpentine was different. They had taken melting points and had been quite satisfied about the purity of their compounds. It could be concluded that reserpine and rescinnamine had similar qualitative effects as blood pressure reducing agents. Methyl reserpate had been found as a metabolic product of reserpine in the body, but they did not know whether it was active.

MR. LEWIS replied. The drugs probably acted as intact molecules, the theory being that they entered the central nervous system and affected certain receptor sites, altering these in some way. The drug was then rapidly degraded and disappeared, leaving the receptor sites altered.

A COMPARATIVE STUDY OF THE HYDROLYTIC AND NON-HYDROLYTIC METHODS FOR THE ASSAY OF SOLANACEOUS DRUGS

By R. E. A. Drey

From the Wellcome Chemical Works, Dartford, Kent

Received June 1, 1958

The results given by the hydrolytic and non-hydrolytic methods of determining the total alkaloidal content are in good agreement for belladonna, hyoscyamus and stramonium; the agreement is fair for *Duboisia leichhardtii* and *Datura sanguinea*, whilst for *Duboisia myoporoides* the non-hydrolytic method gives high values. An improved paper chromatographic system for the separation of the principal tropane alkaloids has been developed; the system was used to determine the individual alkaloidal content of the respective drugs and to provide the appropriate factor for converting titre to percentage of total alkaloid.

A potential source of error in the conventional assay of solanaceous drugs occurs in the final stage of the determination, in which the extract of the total alkaloids is directly titrated against standard acid after removal of the volatile bases by heating at 100°. Errors may arise from incomplete removal of volatile bases, the presence of non-volatile non-alkaloidal bases (for example, tropine and oscine), or the retention of traces of ammonia by the alkaloidal extract[1–7].

To obviate these possible sources of error Reimers proposed a modified method in which the alkaloidal extract is hydrolysed and the liberated carboxylic acids are extracted and titrated against standard alkali[8,9].

This method was subsequently incorporated in the International Pharmacopoeia as one of two alternative procedures for the assay of solanaceous vegetable drugs. Except for *Hyoscyamus muticus*[9] and stramonium[10], however, there is no literature on the results given by the two techniques, and accordingly a comparative study of the direct and hydrolytic methods was undertaken with drugs from commercial sources.

Preliminary Experiments

Loss on Heating of Alkaloids and Amino Alcohols at 100°

In the official assay it is specified that the extract of the bases be heated at 100° and weighed at intervals of 1 hour until two successive weighings do not differ by more than 1 mg. In view of the long periods of heating required by some extracts to reach constant weight, account had to be taken of the effect of prolonged heating of tropane bases at 100°. Information is given in the literature only for atropine and hyoscyamine, and is conflicting. Thus Hardy[11] and DeKay and Jordan[12] stated that atropine is non-volatile at 100°; on the other hand, Schousen[13] and Fricke and Kaufman[14] reported that prolonged heating of atropine and hyoscyamine at this temperature results in loss by decomposition or volatilisation.

Aliquots of standard solutions of the pure bases in chloroform were evaporated to dryness; the residues were heated at 100° for 0, 1, 2 and

4 hours respectively and titrated against standard acid. The respective average losses in weight, expressed in per cent per hour, were as follows:— Hyoscyamine, 0·05; hyoscine, 0·05; valeroidine, 0·15; tigloidine, 3·1; tropine, 2·6; oscine, 4·9.

Determination of Alkaloids by the Hydrolytic Method

A series of preliminary experiments was carried out to establish optimum conditions of hydrolysis, extraction and titration of acids.

Hydrolysis. In the method of Reimers and of the Ph.I. it is specified that the aqueous extract of the alkaloids, which measures not less than 50 ml., be hydrolysed by evaporating with 10 ml. of sodium hydroxide solution until only 10 ml. remains. Using a paper chromatographic procedure it was found, however, that under these conditions both hyoscyamine and hyoscine are completely hydrolysed in 2½ to 3½ minutes, and it is thus possible to reduce appreciably the time required for hydrolysis.

Extraction and titration of carboxylic acids. A systematic study of the variables involved in the hydrolytic method (optimum pH for extraction of carboxylic acids, number of extractions required, etc.,) showed that the procedure of Reimers and of the Ph.I. is satisfactory; the precaution was taken, however, of washing the chloroform-*iso*-propanol extracts with water to minimise the risk of traces of hydrochloric acid being carried over into the extract.

As a result of these experiments the following procedure was adopted: The aqueous solution of the alkaloid or alkaloidal extract was diluted to about 35 ml. with water, mixed with 10 ml. of 2N sodium hydroxide and evaporated for 15 minutes on the water bath. The solution was cooled, neutralised with dilute hydrochloric acid and 0·5 ml. of the acid added in excess. The acid liquid was extracted with four 25 ml. and two 20 ml. portions of a mixture of *iso*propanol (1 part) and chloroform (3 parts), each extract being washed with the same 15 ml. of water. The extracts were combined and the solvent removed by evaporation. The residue was dissolved in 15 ml. of warm water, cooled and titrated with 0·02N sodium hydroxide, using phenolphthalein as indicator.

Results of experiments using known amounts of tropic acid, together with comparative assay results for hyoscyamine sulphate and hyoscine hydrobromide by the hydrolytic and non-hydrolytic methods, are given in Table I. Experiments were also carried out using tropine base; it is seen that the presence of tropine causes no interference in the hydrolytic process.

Chromatography

No combination of paper and developing solvent has been described in the literature for the separation of all the principal tropane alkaloids; in particular the separation of tigloidine from the other alkaloids has not been achieved[15]. Numerous combinations of paper and developing solvent were examined and it was found that a water-saturated mixture of *n*-butanol (3 vol.), *n*-butyl acetate (17 vol.) and glacial acetic acid (8 vol.) used in conjunction with 0·2M potassium chloride-treated papers

gave compact spots and afforded good separations of the amino alcohols and the principal tropane alkaloids, with the exception of hyoscyamine and *nor*hyoscyamine. The R_F values by the descending technique are as follows (bases run as salts). Oscine, 0·06; tropine, 0·09; belladonnine, 0·18; hyoscine, 0·25; hyoscyamine, 0·37; *nor*hyoscyamine 0·38; valeroidine, 0·46; tigloidine, 0·56; *apo*atropine, 0·67. The R_F values are independent of the nature of the anion of the alkaloid or amino alcohol.

TABLE I

RESULTS OF ASSAY BY HYDROLYTIC AND NON-HYDROLYTIC METHODS

Taken	Weight, g.	Method of assay	Per cent recovered
Blank (25 ml. water + 0·5 ml. dilute hydrochloric acid)		B	0
Tropine base	0·1075	C	63·0, 63·9*
	"	B	0
Tropic acid	0·05465	A	101·0, 101·9
	"	B	101·3, 101·7
	0·1070	A	99·4, 100·1
	"	B	99·8, 99·8
Hyoscyamine sulphate, anhyd.	0·1012	C	99·2, 99·5
	"	D	100·5, 100·8
	0·1960	C	97·5, 98·0
	"	D	98·4, 99·2
Hyoscine hydrobromide B.P.	0·1216	C	98·9, 99·3
	"	D	99·2, 99·8
	0·2984	C	99·6, 100·0
	"	D	100·8, 101·0

A Direct titration.
B Extraction from acid solution and titration.
C Chloroform extraction from alkaline solution and titration (non-hydrolytic method).
D Hydrolysis, extraction from acid solution and titration
* Low recovery due to solubility of tropine base in aqueous alkali.

The effect on the chromatograms of the presence of non-alkaloidal volatile bases in the extracts was also examined. The bases were obtained by adding an excess of sodium hydroxide solution to a concentrated extract of the total bases in dilute acid and steam-distilling into an excess of aqueous acid. The volatile bases from the official drugs gave only faint spots in the tropine position, whilst those from *Duboisia myoporoides* and *Duboisia leichhardtii* gave a number of relatively weak spots which did not interfere in the determination of the individual alkaloids.

COMPARATIVE ASSAY PROCESS

Determination of Total Alkaloids

The drug was reduced to No. 60 powder and 25 g. (100 g. in the case of hyoscyamus) extracted in two separate quantities each of 12·5 g. (50 g. in the case of hyoscyamus) using the B.P. 1958 method for Belladonna Herb; all quantities of solvent were increased by 25 per cent. The final chloroform extracts were mixed, evaporated to low bulk and diluted to exactly 50 ml. with chloroform. Two 20 ml. aliquots of this solution, each equivalent to 10 g. of sample (40 g. in the case of hyoscyamus) were taken for the determination of the total alkaloids.

Aliquot 1 ("*non-heated extract*"). The extract was evaporated to dryness and the alkaloids titrated with 0·05N sulphuric acid as in the pharmacopoeial assay. The solution was then diluted to about 35 ml.

with water, hydrolysed with 2N sodium hydroxide and the carboxylic acids extracted and titrated as described above.

Aliquot 2 (*"heated extract"*). The solution was evaporated to dryness in a tared dish, weighed and heated at 100° for 1 hour and re-weighed. The process was continued until two successive weighings did not differ by more than 1 mg. The total alkaloids were then determined as for Aliquot 1.

Determination of Individual Alkaloids and of Alkaloidal Factor

5 ml. of the chloroform extract was evaporated to dryness and the residues dissolved in the following volumes of 0·05N sulphuric acid. *Duboisia myoporoides*, 4 ml.; *Duboisia leichhardtii*, 6 ml.; other drugs, 2 ml. Volumes of this solution, ranging from 5 to 80 μl., and of aliquots of a standard solution of alkaloids were applied to the starting positions of

TABLE II

RESULTS OF ASSAY OF SOLANACEOUS VEGETABLE DRUGS

Drug	Time of heating extract at 100°	Total alkaloids				Individual alkaloids				Factor M
		By direct titration		Hydrolytic method						
		Extract not heated	Extract heated	Extract not heated	Extract heated	Hyoscyamine M.W. = 289·4	Hyoscine 303·3	Valeroidine 241·3	Tigloidine 223·3	
Belladonna herb	1 hr.	0·497	0·486	0·500	0·494	0·45				289·4
Hyoscyamus ..	2 hrs.	0·071	0·053	0·052	0·048	0·021	0·025			296·9
Stramonium ..	1 hr.	0·265	0·260	0·257	0·251	0·13	0·11			295·7
Datura sanguinea ..	1 hr.	0·331	0·304	0·284	0·267	0·05	0·23			300·8
Duboisia myoporoides sample No. 1	8 hrs.	1·68	1·17	0·988	0·913	0·24	0·37	0·15	0·1	279·4
Duboisia myoporoides sample No. 2	10 hrs.	2·34	1·64	1·43	1·11	0·18	0·61	0·35	0·1	277·3
Duboisia leichhardtii ..	10 hrs.	3·36	3·03	3·16	2·94	2·15	0·37			291·4

Whatman's No. 1 filter paper which had previously been impregnated with a 0·2M aqueous potassium chloride solution and blotted between sheets of filter paper[16]. The chromatograms were developed in the downward direction, dried at room temperature for 5 hours and immersed in an aqueous tartaric acid solution of potassium iodobismuthate[17]; the individual alkaloids were then estimated by the technique of matching of spots[18].

The results of the chromatographic examination were used to compute the factor M for converting titre to percentage of total alkaloid.

$$M = \frac{303{\cdot}3\,a + 289{\cdot}4\,b + 241{\cdot}3\,c + 223{\cdot}3\,d}{a + b + c + d}$$

where a, b, c and d are the percentages in the drug of hyoscine, hyoscyamine, valeroidine and tigloidine, respectively. The results of the determinations are given in Table II.

DISCUSSION

The figures in columns 4 and 5 of Table II show that for the official drugs the results given by the hydrolytic and non-hydrolytic methods

are in good agreement. With *Datura sanguinea* and *Duboisia leichhardtii* the agreement is fair, whilst for *Duboisia myoporoides*, even after 8 to 10 hours' heating, the direct titrimetric method gives high results, and this drug should therefore be assayed by the hydrolytic method. It is recommended that the hydrolytic process be used also for *Duboisia leichhardtii* to avoid the need for an unduly long period of heating of the alkaloidal extract. The same remark probably applies to Indian belladonna, which has an exceptionally high content of volatile bases[19] and the extract of which may have to be heated at 100° for periods of up to 6 hours to reach constant weight[20]. Unfortunately no sample of this drug was available to investigate this point.

The discrepancy between the "non-heated" and "heated" hydrolytic values for *Duboisia myoporoides* and *Duboisia leichhardtii* (columns 5 and 6) is most probably due to the presence of small quantities of esters that are slightly volatile at 100°, for example tigloidine base in *Duboisia myoporoides* (*v. supra*), and *iso*butyryltropeine and *d*-α-methylbutyryltropeine in *Duboisia leichhardtii*[21,22].

In the paper chromatographic examination the error in the determination of the individual alkaloids amounted to 10 to 15 per cent; this error, however, had only a small effect on the accuracy of the factor M.

No *apo*atropine was found in any of the drugs that were examined, and only the sample of hyoscyamus gave a spot in the belladonnine position on the chromatograms, but no attempt was made to confirm the identity of this spot.

Acknowledgements. The author wishes to express his thanks to Dr. G. E. Foster for his interest throughout this investigation, and to Messrs. T. & H. Smith Ltd. and C. H. Boehringer Sohn for samples of tigloidine hydrobromide and of *apo*atropine.

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DISCUSSION

The paper was presented by MR. R. E. A. DREY.

THE CHAIRMAN. It appears that the hydrolytic method is of more general application than the non-hydrolytic process.

DR. W. MITCHELL (London). Was the *Duboisia myoporides* botanically identified? He thought that true duboisia did not contain any atropine and was surprised to hear that it contained hyoscyamine. Were the lower results with duboisia by the hydrolytic method due to the minor alkaloids, which were less readily hydrolysed than hyoscyamine and which are contained in all species of duboisia, not being completely hydrolysed? Tigloidine but not valeroidine could be extracted with chloroform from hydrobromic acid solution.

MR. C. A. JOHNSON (Nottingham). The suggested method would no doubt be more satisfactory for Indian belladonna than the old B.P. process. Had assays been carried out on belladonna root which might contain a high proportion of volatile bases?

MR. DREY replied. The duboisia was of commercial grade which he thought was genuine, but it may have been contaminated with some similar material. Using a paper chromatographic procedure he had found that hydrolysis of hyoscine and hyoscyamine was complete in $2\frac{1}{2}$ minutes, but 15 minutes had been allowed for the hydrolysis. Account had to be taken of the possibility that extraction of tigloidine hydrobromide with chloroform might not be complete. Belladonna root had not been included among the drugs examined.

IDENTIFICATION OF SEEDS FROM VARIOUS SPECIES OF *STROPHANTHUS*

By W. G. Thomas and Colin Melville

From the Pharmacy Department, University of Manchester

Received May 29, 1958

In a search for differential specific characters, the outer epidermis of the testa from *Strophanthus* seeds of 15 species has been examined. A "trichome index" has been defined, and the method of its determination described. It enabled the species studied to be divided into six groups.

The genus *Strophanthus* includes over 60 species, the seeds of about 30 of which have been subjected to chemical examination. Since the first study of the pharmacognosy of *Strophanthus* by Elborne[1] in 1887, many workers have searched for characters by which the seeds of one species might be readily distinguished from another. The seeds are morphologically similar, hence much reliance has been placed on chemical tests as a means of distinguishing them. In 1927, Mathiesen[2] studied authentic seeds of several species, checking details of anatomical structure, and searching for diagnostic characters for samples of commercial seed. Results of the sulphuric acid colour test on fourteen species were recorded, showing the variety of colours produced during the course of the reaction on a single species, and the similarity of colours produced in several different species. Fourteen colour tests were investigated by Smelt[3], who recommended the use of four tests to distinguish between *S. kombé* Oliver and *S. emini* Aschers.

The numerous chemical studies of Jacobs, of Stoll and of Reichstein have shown the variation in constituents according to species. The possible use of sarmentogenin in cortisone syntheses caused a period of renewed interest in the genus, during which some confusion between seeds of the various species occurred. Several expeditions explored Africa from 1949 to 1951 to collect as many species as possible[4,5] and the material had been studied botanically by Monachino[6,7], who reviewed the genus, observing polymorphism in species having a wide geographical distribution. Youngken and Simonian[8] illustrated the morphological characters of four species.

Bush and Taylor[9] found the sulphuric acid test to be unreliable and largely dependent on substances other than cardiac glycosides. They developed a paper chromatographic method for the routine semi-micro investigation of the easily hydrolysable glycosides of the seeds, and classified 24 species into three groups. This method was suggested as a test for seed samples to replace the sulphuric acid colour test.

Experimental

Although such structural differences as do occur in the seeds of various species of *Strophanthus* are of degree rather than of kind, methods depending on numerical relationships between tissues, cells or cell inclusions have

not been reported. Fifteen species were examined, showing that the outer epidermis of the testa was the most suitable tissue for numerical differences.

The lengths of trichomes on surface preparations could not be measured because of the dense mass of tangled trichomes in certain species, and experiments were made to determine the most suitable method of preparing a suspension of separated intact epidermal cells. The testa was removed from seeds previously softened in water and portions were disintegrated by several reagents. The reagent selected was an aqueous solution containing 5 per cent chromic acid and 5 per cent sulphuric acid. It was found possible to control the degree of disintegration easily with this reagent, by varying the concentration, time and temperature. The testa was placed in a screw-capped bottle of 15 ml. capacity with 1 ml. of the reagent for 5 hours at 20°. The trichomes were separated by centrifugation, washed until acid free and stained by suspending in 50 per cent v/v aqueous glycerol, containing 0·001 per cent methylene blue. The lengths were measured by micro-projection[10].

Results

Five hundred trichomes from each of 24 samples of disintegrated material were measured, and the results summarised in Table I.

TABLE I

Length of trichomes on testa of strophanthus seeds

Strophanthus kombé Oliver		
Sample number	Seed number	Length (microns)
1	1	60–390–900
	2	50–300–700
	3	50–390–800
	4	40–440–900
	5	70–310–700
2		70–350–800
3		60–380–700
4		60–360–700
5		40–290–700
6		50–400–900
Other species		
S. amboensis Engl. et Pax.		80–185–435–600
S. courmontil Sacleux.		40–160–440–700
S. emini Aschers.		100–345–935–1300
S. gerrardii Stapf.		50–150–330–500
S. grandiflorus (Brown) Gilg.		40–180–400–500
S. gratus Baill.		—
S. hispidus A.P.DC.		50–155–325–500
S. hypoleucus Stapf.		40–140–340–500
S. intermedius Pax.		100–240–500–700
S. nicholsoni Holmes.		200–440–1100–1700
S. preussii Engl. et Pax.		40–140–310
S. sarmentosus A.P.DC.		60–145–275–400
S. speciosus Reber.		30–50–120
S. welwitschii K. Schum.		50–165–415–700

From these data it appeared possible to distinguish certain species by length of the trichome. The number, per cent, of trichomes exceeding a selected critical length was tested, using several levels of the critical length, and termed the 'trichome index'. This is defined as the number, per cent, of trichomes which exceed a certain critical length. The latter is specified by prefix, for example: "100 micron trichome index".

The results of trichome index determinations using several critical lengths are given in Table II.

TABLE II

TRICHOME INDEX AT DIFFERENT CRITICAL LENGTHS

Trichome index	100 μ	200 μ	300 μ	400 μ	500 μ	1000 μ
S. amboensis	98	80	47	23	7	0
S. courmontii	95	72	48	25	7	0
S. emini	99	93	84	77	67	11
S. gerrardii	95	65	24	3	0	0
S. grandiflorus	96	67	20	2	0	0
S. gratus	0	0	0	0	0	0
S. hispidus	96	67	20	2	0	0
S. hypoleucus	93	63	27	6	1	0
S. intermedius	100	89	69	38	15	0
S. kombé	94	80	60	40	20	0
S. nicholsoni	100	100	97	89	80	24
S. preussii	76	17	1	0	0	0
S. sarmentosus	98	52	7	0	0	0
S. speciosus	2	0	0	0	0	0
S. welwitschii	95	76	46	20	5	0
Strophanthus kombé					300 μ	400 μ
Limits of variation between seeds					64–70	45–55
Limits of variation between samples					55–70	35–50

TABLE III

SEPARATION TABLE, USING TRICHOME INDEX (T.I.) TO GROUP *Strophanthus* SPECIES; ALSO COMPARING BUSH AND TAYLOR'S CLASSIFICATION[9], AND COLOURS REPORTED WITH 80 PER CENT SULPHURIC ACID ON THE ENDOSPERM OF SEED SECTIONS

Group	Species	Bush and Taylor's classification	80 per cent sulphuric acid test
I. 1000 μ T.I. exceeds 18	*S. nicholsoni*	2	red
II. 1000 μ T.I. less than 18 400 μ T.I. exceeds 70	*S. emini*	2	red or orange
III. 400 μ T.I. less than 70 300 μ T.I. exceeds 35	*S. amboensis*	3	red or orange
	S. courmontii	3	orange
	S. grandiflorus	—	red or orange
	S. intermedius	3	—
	S. kombé	2	green
	S. welwitschii	3	red
IV. 300 μ T.I. less than 35 200 μ T.I. exceeds 37	*S. gerrardii*	3	red or orange
	S. hispidus	2	green
	S. hypoleucus	2	red or orange
	S. sarmentosus	3	red or orange or green
V. 200 μ T.I. less than 37 100 μ T.I. exceeds 35	*S. preussii*	2	—
VI. 100 μ T.I. less than 35	*S. gratus*	1	red
	S. speciosus	—	red or orange

DISCUSSION

The fifteen species of strophanthus seeds studied were divided into six groups, using the proposed trichome index (Table III). Variations between seeds, and between samples, are shown by the measurements upon *S. kombé* to be less than the variation between the six groups proposed in Table III. The use of this separation table is proposed as an aid to the identification of the seeds from various species of *Strophanthus*.

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DISCUSSION

The paper was presented by Mr. W. G. Thomas.

THE CHAIRMAN. Could the method be applied to powdered seed? He had not found the sulphuric acid test to be unreliable. Was it possible that seeds of *Strophanthus sarmentosus* had been confused with *S. kombé* as he believed the green colour with the latter to be characteristic?

MR. S. G. E. STEVENS (London). It would be difficult to differentiate clearly between *S. kombé* and *S. emini* solely on length of trichomes. Would the treatment have the same effect on the short stubby trichomes as on the tenuous ones?

DR. T. E. WALLIS (London). Insufficient information about the procedure had been given. *S. nicholsoni* would give difficulties because of its matted, twisted trichomes. Those of *S. hespidus* were brittle and were broken off during commercial handling. It would be difficult to measure the length when the trichomes were twisted or overlapped. Did the length include the base embedded in the epidermis? Was the apex always complete? Was the number measured for each sample a chance number or did it represent the total hairs present on the seed? By examination of the endosperm and the crystals, with one exception, he had, not had any difficulty in detecting seeds of other species when present in samples of kombé seeds.

DR. B. P. JACKSON (Sunderland). For kombé, 10 seeds were mentioned, were the other determinations carried out on only one seed? Were they young seeds with hairs probably not fully developed or were they mature seeds?

MR. THOMAS replied. The colour test had been carried out on botanically authenticated samples. The variation with *S. sarmentosus* appeared to be due to geographical variation in the species and there had been work which suggested that there should be one or two new varieties of this species. The suggested method could be applied to the powdered seed. The trichomes had been measured by micro-projection and damaged trichomes would be revealed. He agreed that the groupings were fine and that the method must be used in conjunction with other methods. The short stubby trichomes had survived the treatment. The matted trichomes of *S. nicholsoni* could be seen clearly after applying the method for separation. He agreed that the fragility of the trichomes of *S.*

hespidus would give rise to difficulties in commercial samples. The length measured included the base itself. The actual number of hairs on the seed had not been determined. The figures quoted were the results from many hundreds of seeds. It was considered that 500 was the minimum number of trichomes which must be measured from one seed.

THE FUNCTIONAL GROUPINGS OF CUCURBITACIN E (α-ELATERIN)

By J. N. T. Gilbert and David W. Mathieson

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Received June 1, 1958

In addition to the widespread use of cucurbits for their food value there remain many suggestions that members of the family *Cucurbitaceae* are useful for the alleviation of a variety of complaints. Thus claims have been made[1], and refuted[2], that several species viz. *Bryonia cretica*, *Cephelandra indica* and *Momordica charantia* exert a hypoglycaemic action in diabetic patients. Extracts of *Bryony*, *Colocynth* and *Elaterium* have been shown to possess a tumour necrosing action[3] whilst extracts of *Cucumis melo*[4] and *C. sativus*[5] have been claimed to inhibit the growth of fungi. Findings of this sort have not as yet led to important applications and it is only of the purgative action of certain members of this family that any medicinal use is still made. Such use is ancient and mention of *Citrullus colocynthis* is to be found in an Anglo-Saxon herbal[6] (useful "for stirring of the inwards"). The same drug is likewise to be found in the Papyrus Ebers[7] and is believed by some authorities[8] to be the fruit of Elisha's miracle[9]. Interest in biological activity of this sort however is waning for obvious reasons and revival of interest in certain constituents of this family centres on the first-mentioned use of the cucurbit as an article of diet. In Europe, Africa and elsewhere, the occurrence of bitterness in cultivated cucurbits presents a problem not only from the economic viewpoint but also as something of a hazard to human health[10]. Systematic search for the bitter principles involved has led to the isolation of eleven toxic substances provisionally called the Cucurbitacins and numbered alphabetically[11]; these represent a new class of triterpenes characterised by their

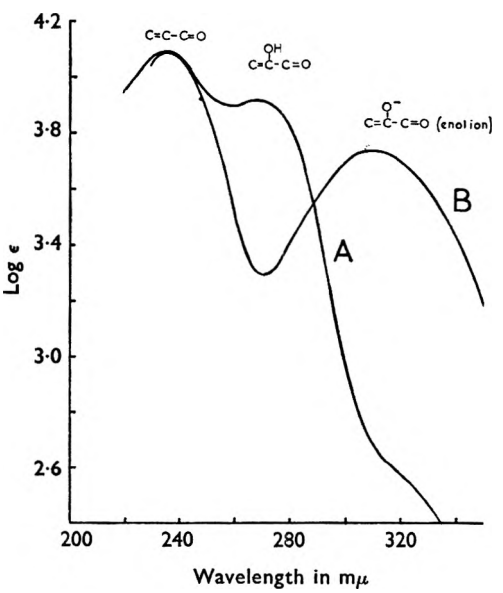


Fig. 1. Ultra-violet absorption spectrum of A, cucurbitacin E in ethanol; B, cucurbitacin E in 0·1M Na_2CO_3.

unusually high oxygen content. Cucurbitacin E (α-elaterin) is one member of the above list which constitutes approximately 15 per cent by weight of the crude drug elaterium. It is normally present as a glycoside in many cucurbitaceous fruits but in the case of *Ecballium elaterium* (squirting cucumber) for example, is rapidly hydrolyzed to the genin by the enzyme elaterase[12]. Not all species contain this enzyme—*Citrullus* being one such—and it is of interest to note that this is the probable reason for the failure of later workers[13] to repeat the original isolation by Power and Moore[14] of α-elaterin from *Citrullus colocynthis*.

Structural studies on Cucurbitacin E (α-elaterin) have been carried out in these laboratories and full details will be published elsewhere. The following summarises the position with regard to the functional groupings of this substance.

Accurate determination of the molecular weight[15] has allowed the assignment of a revised molecular formula $C_{32}H_{44}O_8$. Analysis indicates the presence of one *O*-acetyl group and in the light of what follows, the corollary may be added that Cucurbitacin E is probably pentacyclic. Since this substance forms but few, if any, normal derivatives of carbonyl function and hydroxyl group, evidence for the presence of such is mainly spectroscopic. Figure 1 shows the ultra-violet absorption spectrum of Cucurbitacin E and the assignment of the bands. Thus Cucurbitacin E gives a positive ferric chloride reaction and with the benzene diazonium ion, a red colour. It has an apparent pKa value in 50 per cent ethanol of 10·8. With acetic anhydride-sodium acetate two acetyl groups may be inserted in the molecule; one a normal *O*-acetyl group, the other an enol acetate of the above 1 : 2-diketone. The resulting Cucurbitacin E acetate has lost the band characteristic of the 1 : 2-diketone and retains that due to the enone system with the intensity approximately doubled. (Fig. 2).

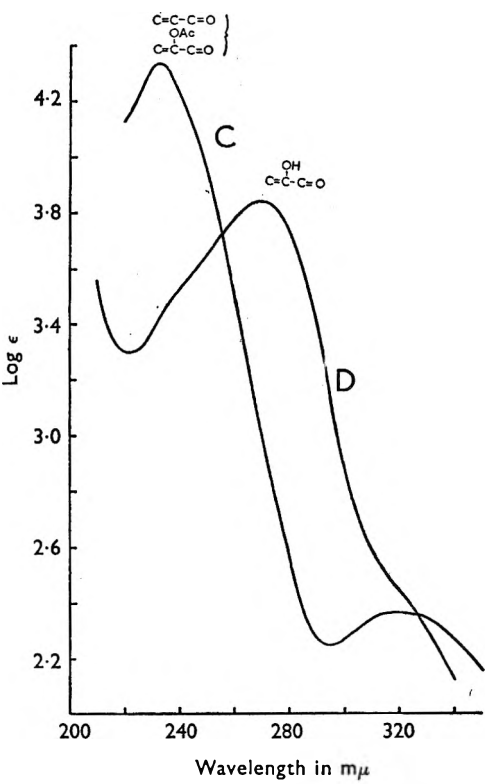


FIG. 2. Ultra-violet absorption spectrum in ethanol of C, cucurbitacin E acetate; D, dihydrocucurbitacin E*.

*In calculating values of ε, a molecular weight of 559 has been assumed.

On the other hand, a "dihydrocucurbitacin E" shows no enone band and only that characteristic of the 1 : 2-diketone.

Confirmation of these oxygen functions is to be found in the infra-red spectrum. (Fig. 3).

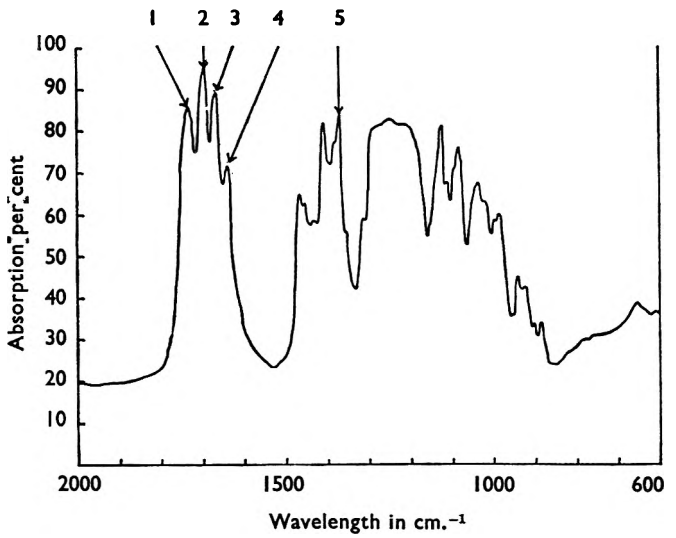
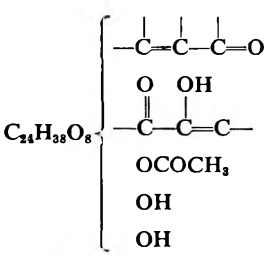


FIG. 3. Infra-red spectrum of cucurbitacin E. Solvent, chloroform. 1, Acetyl carbonyl; 2, 2 × carbonyl; 3, enolic 1:2-diketone; 4, double bond $\alpha\beta$ to carbonyl; 5, acetoxy group.

Of the hydroxyl groups present in Cucurbitacin E, one is already acetylated; a second, as noted above, may be so esterified whilst a third fails to react in this wise. The third "inert" hydroxyl is most easily shown by a band in the infra-red region at 3460 cm.$^{-1}$ and characteristic of associated hydroxyl groups. Zerewitinoff determination of active hydrogen indicates three hydroxyl groups in Cucurbitacin E one of these is the enolic hydroxyl from the 1 : 2-diketone. Thus far seven of the eight oxygen atoms may be assigned their function.



When Cucurbitacin E is treated with cold sodium hydroxide several changes take place in the molecule. Firstly the acetyl group is split off and secondly the $\alpha\beta$-unsaturated ketone undergoes what is probably a reversed aldol shift (Rearrangement A). The intermediate product which results (elateridine) is as yet inadequately characterised but on further

treatment with caustic alkali at 100°, yields the crystalline ecballic acid in which a benzilic acid change of the 1:2-diketone has further taken place (Rearrangement B). Acetoin has recently been shown to be a product of alkaline isomerization and the overall changes resolve themselves thus[15]

$$C_{32}H_{44}O_8 \downarrow 3H_2O$$

$$C_{26}H_{38}O_7 + CH_3CHOH{\cdot}COCH_3 + CH_3CO_2H$$

Ecballic acid — Acetoin — Acetic acid

Ecballic acid yields iodoform on treatment with iodine and sodium hydroxide and the presence of a methyl ketone is thus indicated. It arises as a result of rearrangement A. The formation of a bis-2:4-dinitrophenyl hydrazone indicates a second carbonyl group which we believe is also present in the parent Cucurbitacin E: it thus accounts for the eighth oxygen. Rearrangement B (the benzilic acid change) is the source of the carboxylic acid group of ecballic acid.

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DISCUSSION

The short communication was presented by DR. D. W. MATHIESON.

THE CHAIRMAN. How had the Authors prepared the Cucurbitacin E? Did any other curcubitacin correspond with β-elaterin? What was the difference in the structure of α and β-elaterin?

DR. J. B. STENLAKE (Glasgow). An infra-red frequency a little higher than normal had been quoted for a hydroxyl group, might this be a tertiary hydroxyl which would account for its being inert?

DR. SZINAI (Israel). The 1690 line was stated to be due to two carbonyl groups. Which carbonyl group could give rise to this absorption? As one dehydrogenation product of α-elaterin was 1 : 2 : 8-trimethylphenanthrene, was not a tetracyclic triterpene more likely the basic structure?

DISCUSSION

MR. S. G. E. STEVENS (London). Had the Authors truly compensated for the solvent? Had they tried carbon disulphide which would give fewer bands in the region investigated?

DR. MATHIESON replied. Crude elaterium was the sediment from the expressed juice of the unripe cucumber. After percolation with light petroleum to remove chlorophyll and then with chloroform, α-elaterin was obtained by crystallisation from a crude crystalline principle which appeared in the chloroform extract. There were five or six closely related cucurbitacins. α and β-Elaterin were similar and were probably triterpenoids with seven or eight oxygen atoms. The band at 3460 $cm.^{-1}$ was probably the tertiary hydroxyl but the presence of eight oxygen atoms might affect the assignments of the bands. When examined using a grating system, the 1690 band was seen to consist of two discreet bands. Two carbonyls therefore became more likely. Pentacyclic triterpenes normally gave both phenanthrenes and napthalenes on dehydrogenation. α-Elaterin was insoluble in carbon disulphide. As a double beam instrument was used, solvent correction was automatically applied.

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