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**TRANSACTIONS OF THE
BRITISH PHARMACEUTICAL CONFERENCE
CENTENARY MEETING
LONDON 1963**

17 BLOOMSBURY SQUARE, LONDON, W.C.1

JOURNAL OF PHARMACY AND PHARMACOLOGY

TRANSACTIONS OF THE
BRITISH PHARMACEUTICAL CONFERENCE

EDITOR: D. W. MATHIESON, B.Sc., Ph.D., F.R.I.C.
PRESS EDITOR: J. R. FOWLER, B.Pharm., F.P.S.

17, BLOOMSBURY SQUARE, LONDON, W.C.1

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BRITISH PHARMACEUTICAL CONFERENCE

CENTENARY MEETING, LONDON, 1963

REPORT OF PROCEEDINGS

OFFICERS:

President:
C. W. Maplethorpe, M.Sc., F.P.S., F.R.I.C., M.I.Chem.E., Ware.

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

Vice-Chairmen:
R. R. Bennett, B.Sc., F.P.S., F.R.I.C., Eastbourne.
H. Deane, B.Sc., F.P.S., F.R.I.C., Sudbury.
H. Humphreys Jones, F.P.S., F.R.I.C., Liverpool.
T. E. Wallis, D.Sc., F.P.S., F.R.I.C., F.L.S., London.
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H. Berry, B.Sc., Dip.Bact. (Lond.), F.P.S., F.R.I.C., Eastbourne.
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K. Bullock, M.Sc., Ph.D., F.P.S., F.R.I.C., Manchester.
F. Hartley, B.Sc., Ph.D., F.P.S., F.R.I.C., London.
G. E. Foster, B.Sc., Ph.D., F.R.I.C., Dartford.
H. Treves Brown, B.Sc., F.P.S., London.
W. H. Linnell, D.Sc., Ph.D., F.P.S., F.R.I.C., London.
D. C. Garratt, D.Sc., Ph.D., F.R.I.C., Nottingham.
J. C. Hanbury, M.A., B.Pharm., F.P.S., F.R.I.C., Ware.

Honorary Treasurer:
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Honorary General Secretaries:
E. F. Hersant, B.Pharm., Ph.D., F.P.S., F.R.I.C., London.
D. Train, M.C., Ph.D., F.P.S., F.R.I.C., A.M.I.Chem.E., 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*).

*A. Aldington, M.P.S., London.
A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C., London.
*J.C. Bloomfield, M.P.S., Portsmouth.
*Miss M. A. Burr, M.P.S., Nottingham.
K. R. Capper, Ph.D., B.Pharm., F.P.S., D.I.C., London.
D. W. Hudson, M.P.S., Hove.
E. Shotton, B.Sc., Ph.D., F.P.S. F.R.I.C., London.
R. E. Stuckey, D.Sc., Ph.D., F.P.S., F.R.I.C., London.
W. T. Wing, F.P.S., Newcastle.

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

PROCEEDINGS OF CONFERENCE
LONDON, 1963

THE OPENING SESSION

The opening session of the Conference was held in the Grand Hall of the Connaught Rooms on Monday, September 2, with Mr. C. W. Maplethorpe, President of the Conference (President of the Pharmaceutical Society), in the Chair. On the platform were the Chairman of the Conference (Mr. H. G. Rolfe), the Minister of Health (Mr. Enoch Powell), the Chairman (Mr. A. Aldington) and Secretary (Dr. K. R. Capper) of the Local Committee, the Conference Treasurer and the Honorary General Secretaries, together with members of the Conference Executive.

The President introduced the Minister of Health, who extended to the Conference "a most cordial welcome on behalf of Her Majesty's Government". On the motion of the President a vote of thanks to the Minister for his address was carried by acclamation.

The President then handed over further conduct of the Conference to the Chairman (Mr. H. G. Rolfe), who delivered his address entitled "A History of the Conference", which is printed in the Supplement to the *Journal of Pharmacy and Pharmacology*, **15**, 9*T*–42*T*.

On the proposition of Mr. H. Treves Brown, the Conference accorded a vote of thanks to the Chairman for his address.

GUILDHALL RECEPTION

On the evening of Monday, September 2, the Conference members were the guests of the Lord Mayor and Corporation of London. In the absence of the Lord Mayor abroad, guests were received by the Lord Mayor locum tenens, Sir Noel Bowater (Lord Mayor, 1953–4).

THE SCIENCE SESSIONS

Meetings were held on Monday, Wednesday and Friday, September 2, 4 and 6, at the Connaught Rooms, the Chairman and past Chairmen presiding. During the sessions the following 35 papers were presented.

1. Sterilisation of Colchicine Injection. By G. Smith, B.Sc., F.P.S., J. M. Bullivant, B.Pharm., F.P.S. and P. H. Cox, M.P.S.
2. Some Antipyretics Related to Aspirin and Phenacetin. By J. A. Baker, B.Sc., Ph.D., J. Hayden, M.A., P. G. Marshall, D.Sc., F.R.I.C., C. H. R. Palmer, B.Pharm., M.P.S., and T. D. Whittet, B.Sc., Ph.D., F.P.S., F.R.I.C., D.B.A.
3. An Accelerated Storage Test with Programmed Temperature Rise. By A. R. Rogers, B.Pharm., B.Sc., Ph.D., F.P.S., F.R.I.C.
4. The Instrumentation of a Rotary Tablet Machine. By E. Shotton, B.Sc., Ph.D., F.P.S., F.R.I.C., J. J. Deer and D. Ganderton, B.Pharm., Ph.D., M.P.S.
5. The Evaluation of Canada Balsam. By S. B. Challen, B.Pharm., B.Sc., Ph.D., F.P.S., F.L.S.
6. Chemical Studies of the Leaves and Inflorescences of *Digitalis purpurea* L. and of Allied Species. By P. S. Cowley, B.Pharm., Ph.D. and J. M. Rowson, M.Sc., Ph.D., F.P.S., F.L.S.
7. The Use of Orthogonal Functions to Correct for Irrelevant Absorption in Two Component Spectrophotometric Analysis. By A. L. Glenn, B.Pharm., B.Sc., Ph.D., F.P.S.
8. Charge Delocalisation in Relation to Neuromuscular Blocking Activity of Certain Tetra-alkylammonium Compounds. By B. Collier, B.Sc. and K. A. Exley, M.D.
9. The Reaction of Semicarbazide with Collagen. By G. C. Wood, Ph.D.
10. An Experimental Determination of the Internitrogen Distance in Some Bis-quaternary Ammonium Ganglionic and Neuromuscular Blocking Agents. By P. H. Elworthy, B.Pharm., Ph.D., M.P.S., A.R.I.C.
11. On the Relationship Between the Effect of Phenol on the Oxygen Uptake and the Viability of *Penicillium notatum* Spores. By N. M. Chauhan, B.Pharm., M.P.S., Stella M. Rivers, B.Sc., F.P.S. and V. Walters, B.Pharm., Ph.D., F.P.S.
12. Heat and Gamma-Radiation Resistance of *Bacillus megaterium* Spores. By A. Tallentire, M.Sc., Ph.D., F.P.S. and C. O. Chiori, M.Sc.

13. The Evaluation, from Extinction Data, of the Inactivation of Bacteriophage by Chemical Agents. By A. M. Cook, B.Pharm., Ph.D., Dip.Bact., F.P.S., F.R.I.C. and W. R. L. Brown, B.Pharm., Ph.D., F.P.S.
14. The *Mitragyna* Species of Ghana. The Alkaloids of the Leaves of *Mitragyna stipulosa* (D.C.) O. Kuntze. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C., E. J. Shellard, B.Pharm., Ph.D., F.P.S., F.R.I.C., F.L.S. and A. N. Tackie, B.Pharm., Ph.D.
15. The *Mitragyna* Species of Ghana. The Alkaloids of the Leaves of *Mitragyna ciliata* Aubr. et Pellegr. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C., E. J. Shellard, B.Pharm., Ph.D., F.P.S., F.R.I.C., F.L.S. and A. N. Tackie, B.Pharm., Ph.D.
16. *Datura sanguinea* R. and P., its Stem and Leaves. By T. E. Wallis, D.Sc., F.P.S., F.R.I.C., F.L.S. and Mirjana Konjovic.
17. A Dependence on Water Content of Bactericidal Efficiency of Gamma-Radiation. By A. Tallentire, M.Sc., Ph.D., F.P.S., N. A. Dickinson, B.Pharm., M.P.S. and J. H. Collett, B.Sc., M.P.S.
18. A Common Antigen in the Cell Walls of Three Lysozyme-sensitive Bacteria. By D. Wiseman, B.Sc., M.P.S.
19. The Action of Tetracycline and Chloramphenicol Alone and in Admixture on the Growth of *Escherichia coli.* By E. R. Garrett, B.S., M.S., Ph.D. and M. R. W. Brown, M.Sc., Ph.D., M.P.S., M.I.Biol.
20. The Use of Membrane Filters in the Enumeration of Damaged *Escherichia coli.* By N. D. Harris, B.Pharm., Ph.D., F.P.S., D.I.C. and J. P. Richards, B.Sc., Ph.D., M.P.S.
21. The Influence of the Nature of the Recovery Medium on the Apparent Viability of Phenol-treated Bacteria. By N. D. Harris, B.Pharm., Ph.D., F.P.S., D.I.C.
22. Phase Solubility Analysis: An Evaluation of the Technique. By D. C. Garratt, D.Sc., Ph.D., Hon.M.P.S., F.R.I.C., C. A. Johnson, B.Pharm., B.Sc., F.P.S., F.R.I.C. and R. E. King, A.R.I.C.
23. The Colorimetric Determination of Small Amounts of Iodide, with Special Reference to the Determination of Iodide in the Presence of Organically-bound Iodine. By R. E. A. Drey, B.Sc., F.R.I.C.
24. The Estimation of Morphine, Codeine and Thebaine in Opium and in Poppy Latex by Paper Chromatography. By J. W. Fairbairn, B.Sc., Ph.D., F.P.S., F.R.I.C., F.L.S. and Gamila Wassel, B.Pharm.
25. Ionisation Constants of some Penicillins and of their Alkaline and Penicillinase hydrolysis Products. By H. D. C. Rapson, B.Sc., Ph.D., D.I.C., A.R.I.C. and A. E. Bird, B.Sc., A.R.I.C.
26. A Chromatographic Method for the Determination of Phenothiazine. By A. Holbrook, F.R.I.C., F. S. Barlow and F. Bailey.
27. Multiple Spot Phenomena Using Thin Layer Chromatography of Pure Organic Bases. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C. and N. Choulis, B.Pharm.
28. Spectroscopic Studies of the Reaction of Hydroxylated Promazines and Related Compounds with Sulphuric Acid. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C. and S. H. Curry, B.Pharm., M.P.S.
29. Active Sites in Stereoselective Adsorbents as Models of Drug Receptors and Enzyme Active Sites. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C. and H. Z. Youssef, B.Pharm.
30. The Structures of the Alkaloids from *Mitragyna* Species of Ghana. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C. and A. N. Tackie, B.Pharm., Ph.D.
31. A Rapid Method for the Determination of Griseofulvin in Fermenter Broth. By A. Holbrook, F.R.I.C., F. Bailey and Greta M. Bailey.
32. A Quantitative Chromatographic Method for the Determination of Purity of Griseofulvin. By A. Holbrook, F.R.I.C., F. Bailey and Greta M. Bailey.
33. The *Mitragyna* Species of Ghana. The Anatomy of the Leaves of *Mitragyna stipulosa* (D.C.) O. Kuntze and *Mitragyna ciliata* Aubr. et Pellegr. By E. J. Shellard, B.Pharm., Ph.D., F.P.S., F.R.I.C., F.L.S. and Parirokh Shadan, Ph.D.
34. The Structure of Cascarosides A and B. 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 S. Simic, B.Pharm.
35. A New Method of Streptomycin Chromatography and its Use in the Examination of the Reaction between Streptomycin and Ammonia. By T. E. Couling, F.R.I.C. and R. Goodey, F.R.I.C.

REPORT OF PROCEEDINGS

THE CONFERENCE LECTURE

A lecture on "Toxic Hazards from Drugs" was given on Tuesday, September 3, by Dr. J. M. Barnes. The Chairman presided. The lecture is printed in the Supplement to the *Journal of Pharmacy and Pharmacology*, **15**, 75*T*–91*T*.

THE SYMPOSIUM SESSION

A symposium on "Pharmaceutical Aspects of Fine Particles and their Evaluation" was held on Thursday, September 5. The Chairman presided. The introductory papers were by Mr. K. A. Lees and Dr. H. Heywood. The meeting is reported in the Supplement to the *Journal of Pharmacy and Pharmacology*, **15**, 43*T*–74*T*.

PROFESSIONAL SESSIONS

With the President of the Conference, Mr. C. W. Maplethorpe, in the Chair, professional sessions were held on the mornings of Wednesday, September 3, when Professor M. J. Rodman read a paper on "Accidental Poisoning", and Friday, September 6, when Messrs. D. A. Norton and F. H. Oliver read introductory papers to the subject "Education for Pharmaceutical Practice—A Teaching Viewpoint". Full reports of the papers and discussions were published in the *Pharmaceutical Journal*, 1963, **191**, 249–255; 275–276; 315–322, 332.

THE CLOSING SESSION

The closing session of the Conference was held on Friday, September 6, in the Grand Hall of the Connaught Rooms, the Chairman presiding.

Vote of Thanks to Local Committee

The Chairman called on Mrs. Estelle Leigh to propose a vote of thanks to the Local Committee. This was seconded by the President. Mr. A. Aldington (Chairman of the Local Committee) replied to the vote of thanks.

Annual Report

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

Your Executive has pleasure in presenting the one-hundredth Annual Report.

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

Obituary.—The Executive report with deep regret the death since the last meeting, of Mr. Granville Shaw, President of the Conference in 1956; and Mr. A. D. Powell, Chairman of the Conference in 1950.

Reports on 1962 Meeting.—The Report of the meeting of the Conference in Liverpool in 1962 together with the science papers and discussions, and the Conference Lecture were published as a supplement to the 14th 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 135.

Conference Papers, 1963.—Fifty papers were submitted; twenty-six full papers and nine short communications were accepted for presentation. The Executive thanks the authors of these papers and also the authors of the papers presented to the Symposium and the Professional Sessions for their contributions. The Executive is grateful to the Editor of the *Journal of Pharmacy and Pharmacology* and to the Editor of *The Pharmaceutical Journal* for making galley proofs of the papers available before this meeting.

Conference Lecture.—The 1963 Conference Lecture was delivered by Dr. J. M. Barnes, Head of the Toxicology Research Unit of the Medical Research Council, and the Executive wishes to record thanks to him for his contribution to the Conference.

Journal of Pharmacy and Pharmacology.—The Executive has been represented on the Editorial Board by the Chairman, the immediate past Chairman and the Senior Honorary General Secretary.

Future Meetings.—An invitation will be presented at this meeting for the Conference to meet in Edinburgh during the week commencing September 14, 1964. Your Executive has provisionally accepted an invitation to hold the

Conference in Cardiff in 1965 during the week commencing September 6, and in Manchester in 1966 during the week commencing September 5. Several branches of the Society have made preliminary enquiries regarding the possibility of entertaining the Conference in future years and the Executive is grateful for all these offers of hospitality.

CONSTITUTION AND RULES.—The revised Rules of the Conference were approved at the Annual Meeting in Liverpool in 1962, and come into force at the conclusion of the present Annual Meeting. Your Executive proposes that the annual subscription for elected members of the Conference shall be one guinea.

OFFICERS AND EXECUTIVE OF THE CONFERENCE.—Your Executive has nominated the following Officers for 1963–64.

Chairman: W. Mitchell; *Honorary Treasurer:* J. M. Rowson; *Honorary General Secretaries:* E. F. Hersant and D. Train.

In accordance with Rule 6 the Executive has nominated the following nine persons for election by this meeting as members of the Executive: A. H. Beckett, G. Bryan, S. Durham, D. W. Hudson, C. A. Johnson, J. J. Lewis, H. D. C. Rapson, E. Shotton and G. Sykes.

No other nominations have been received.

Further, in accordance with the revised constitution the following five past-Chairmen will serve on the Executive:—H. Treves Brown, W. H. Linnell, D. C. Garratt, J. C. Hanbury and H. G. Rolfe.

The above persons together with the President of the Conference (the President of the Pharmaceutical Society of Great Britain), 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 1963–64.

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

Mr. T. Reid proposed the acceptance of the report and the election of officers of the Conference for the ensuing year. Mr. S. Powlson seconded.

Dr. W. Mitchell thanked the Conference on behalf of the newly-elected officers.

TREASURER'S REPORT

During the financial year ended December 31, 1962, the Local Committee Fund loan of £250 was repaid by the Liverpool Local Committee. This Fund was not called upon by the London Local Committee. A donation of £50 was received from the Liverpool Local Committee which was much appreciated.

For the first time a grant was made to a young author of a Science Paper to enable him to attend the Conference at Liverpool.

The subscriptions of the elected members, including a composition fee from the Pharmaceutical Society of Northern Ireland, amounted to £215 and were credited to the account of the *Journal of Pharmacy and Pharmacology*.

J. M. ROWSON,
Honorary Treasurer.

PLACE OF MEETING FOR 1964

Mr. E. Knott on behalf of the Edinburgh and S.E. Scottish Branch of the Society extended an invitation to hold the Conference in Edinburgh in 1964. Mr. J. G. Coleman proposed that the invitation be accepted, and the President seconded. The vote was put to the meeting and unanimously carried.

VOTE OF THANKS TO CHAIRMAN

Mr. H. Burlinson proposed a vote of thanks to the Chairman.

Dr. A. R. Rogers seconded. The vote was put to the meeting by the President and carried with acclamation.

Mr. Rolfe briefly responded.

REPORT OF PROCEEDINGS

BRITISH PHARMACEUTICAL CONFERENCE

Inaugural Meeting held at Newcastle upon Tyne in 1863*

Years	*Places of Meeting*	*Chairmen*	*Local Secretaries*
1923	London ..	F. W. GAMBLE	W. J. U. WOOLCOCK, C.B.E.
1924	Bath	E. WHITE, B.Sc., F.I.C.	P. J. THOMPSON W. H. HALLETT
1925	Glasgow ..	E. 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
1930	Cardiff ..	J. T. HUMPHREY	J. MURRAY
1931	Manchester ..	J. H. FRANKLIN	R. G. EDWARDS
1932	Aberdeen ..	H. SKINNER	H. M. DUGAN
1933	London ..	C. H. HAMPSHIRE, C.M.G., M.B., B.S., B.Sc., F.R.I.C.	H. N. LINSTEAD
1934	Leeds	C. H. HAMPSHIRE, C.M.G., M.B., B.S., B.Sc., F.R.I.C.	G. C. CRUMMACK J. F. SIMON
1935	Belfast ..	F. W. CROSSLEY-HOLLAND, L.M.S.S.A.	D. L. KIRKPATRICK
1936	Bournemouth	H. DEANE, B.Sc., F.R.I.C.	V. J. SCAMPTON
1937	Liverpool ..	T. E. LESCHER, O.B.E.	W. E. HUMPHREYS
1938	Edinburgh ..	J. RUTHERFORD HILL, O.B.E.	C. G. DRUMMOND
1939	Birmingham ..	J. RUTHERFORD HILL, O.B.E.	D. J. RUSTON
1940	London ..	H. HUMPHREYS JONES, F.R.I.C.	———
1941	London ..	A. R. MELHUISH	———
1942	London ..	T. E. WALLIS, D.Sc., F.R.I.C., F.L.S.	———
1943	London ..	T. E. WALLIS, D.Sc., F.R.I.C., F.L.S.	———
1944	London ..	H. BRINDLE, B.Sc., F.R.I.C.	———
1945	London ..	H. BRINDLE, B.Sc., F.R.I.C.	———
1946	London ..	B. A. BULL, A.R.I.C.	———
1947	Torquay ..	B. A. BULL, A.R.I.C.	T. D. EVANS
1948	Brighton ..	N. EVERS, B.Sc., Ph.D., F.R.I.C.	A. WILSON
1949	Blackpool ..	N. EVERS, B.Sc., Ph.D., F.R.I.C.	R. VARLEY T. A. DURKIN
1950	Glasgow ..	A. D. POWELL, F.R.I.C.	A. OFFICER
1951	Harrogate ..	H. BERRY, B.Sc., 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.	D. 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
1959	Bournemouth	H. TREVES BROWN, B.Sc.	D. F. SMITH
1960	Newcastle upon Tyne	W. H. LINNELL, D.Sc., Ph.D., F.R.I.C.	A. McGUCKIN
1961	Portsmouth ..	D. C. GARRATT, D.Sc., Ph.D., F.R.I.C.	N. L. BANKS
1962	Liverpool ..	J. C. HANBURY, M.A., B.Pharm., F.R.I.C.	D. L. REES
1963	London ..	H. G. ROLFE, B.Sc., F.R.I.C.	K. R. CAPPER

* For details of Presidents and Local Secretaries prior to 1923 see Report of Proceedings for 1962.

Honorary Treasurers (One)

1863 to 1870, H. B. BRADY, F.R.S.
1870 to 1877, G. F. SCHACHT
1877 to 1844, C. EKIN
1884 to 1888, C. UMNEY, F.I.C.
1888 to 1890, W. MARTINDALE
1890 to 1893, R. H. DAVIES, F.I.C.
1893 to 1898, J. MOSS, F.I.C.
1898 to 1912, J. C. UMNEY
1912 to 1925, D. LLOYD HOWARD, J.P.
1925 to 1927, R. R. BENNETT, B.Sc., F.R.I.C.
1927 to 1934, F. W. CROSSLEY-HOLLAND, L.M.S.S.A.
1934 to 1936, T. E. LESCHER, O.B.E.
1936 to 1940, A. R. MELHUISH
1940 to 1947, T. MARNES
1947 to 1952, G. R. BOYES, L.M.S.S.A., B.Sc., F.R.I.C.
1952 to 1953, H. DAVIS, C.B.E., B.Sc., Ph.D., F.R.I.C.
1953 to 1958, H. TREVES BROWN, B.Sc.
1958 to 1962, H. G. ROLFE, B.Sc., F.R.I.C.
1962 to , J. M. ROWSON, M.Sc., Ph.D., F.L.S.

Honorary General Secretaries (Two)

1863 to 1880, Prof. J. ATTFIELD, Ph.D., F.R.S.
1863 to 1871, R. REYNOLDS
1871 to 1884, F. BENGER
1880 to 1882, M. CARTEIGHE
1881 to 1886, S. PLOWMAN, F.R.C.S.
1884 to 1890, J. C. THRESH, M.B., D.Sc.
1886 to 1901, W. A. H. NAYLOR, F.I.C.
1890 to 1903, F. RANSOM
1903 to 1909, E. WHITE, B.Sc., F.I.C.
1901 to 1921, E. SAVILLE PECK, M.A.
1909 to 1919, H. FINNEMORE, B.Sc., F.R.I.C.
1912 to 1923, R. R. BENNETT, B.Sc., F.R.I.C.
1919 to 1929, C. H. HAMPSHIRE, C.M.G., M.B., B.S., B.Sc., F.R.I.C.
1923 to 1927, F. W. CROSSLEY-HOLLAND, L.M.S.S.A.
1927 to 1944, C. E. CORFIELD, B.Sc., F.R.I.C.
1929 to 1947, G. R. BOYES, L.M.S.S.A., B.Sc., F.R.I.C.
1944 to 1953, H. TREVES BROWN, B.Sc.
1947 to 1958, H. G. ROLFE, B.Sc., F.R.I.C.
1953 to E. F. HERSANT, B.Pharm., Ph.D. F.R.I.C.
1958 to D. TRAIN, M.C., B.Pharm., B.Sc., Ph.D., F.R.I.C., A.M.I.Chem.E.

BRITISH PHARMACEUTICAL CONFERENCE

INCOME AND EXPENDITURE ACCOUNT, 1962

Expenditure	£	s.	d.	*Income*	£	s.	d.
Gavel—memento to Host Branch ..	9	10	0	Interest on 2½% Consols	40	5	0
Replica of Chairman's Badge, engraving, etc.	7	19	3	Interest on 3% Savings Bonds ..	6	0	0
Engraving Sports Trophies		10	0	Interest on 3% Exchequer Stock ..	15	0	0
Secretaries' Expenses	62	10	5	Interest on Bank Deposit Account ..	4	18	10
Expenses of Speakers	50	17	2	Donation from Pharmaceutical Society of Northern Ireland ..	25	0	0
Honorarium to Conference Lecturer	26	5	0	Donation from Pharmaceutical Society of Ireland	25	0	0
Income Tax	25	5	8	Liverpool Local Committee Donation	50	0	0
Cheque Book	–	–	–	Deficit carried to Accumulated Fund	36	11	0
Expenses of Young Author of Conference Paper	19	17	9				
Surplus carried to Accumulated Fund	–	–	–				
	£202	15	3		£202	15	3

BALANCE SHEET AT DECEMBER 31, 1962

Liabilities	£	s.	d.	*Assets*	£	s.	d.
Accumulated Fund, as at 31.12.61 ..	2,069	0	4	Investments at cost:			
Deduct: Deficit, 1962	36	11	5	£1,610 2½% Consols	1,250	0	0
(*Add:* Surplus, 1961)				200 3% Savings Bonds 1960–70 ..	200	0	0
				£500 3% Exchequer Stock 1962–63	473	4	10
	2,032	8	11	(Total market value at December 31, 1962: £1,401, 1961: £1,253)			
Creditor		10	6		1,923	4	10
Local Committee Fund	250	0	0	Stock of Replicas (3) of Chairman's Badge	22	1	0
				Loan to Local Committee	–	–	–
				Cash at Bank— Deposit Account . ..340 4 4			
				Less: Overdrawn on Current Account 2 10 9	337	13	7
	£2,282	19	5		£2,282	19	5

Audited and found correct

T. HESELTINE
T. C. DENSTON

April 30, 1963

BRITISH PHARMACEUTICAL CONFERENCE

REVISED CONSTITUTION AND RULES*

1. The British Pharmaceutical Conference is an organisation associated with the Pharmaceutical Society of Great Britain, which exists for the purpose of discussing subjects relative to the science of pharmacy, promoting friendly reunion among pharmacists and those interested in pharmacy and generally furthering the objects sought to be obtained under the Royal Charters granted to the Pharmaceutical Society of Great Britain. The Conference shall meet annually.

2. The Conference shall consist of:

(*a*) Fellows, members, honorary fellows, honorary members and registered students of the Pharmaceutical Society of Great Britain;

(*b*) members of the Pharmaceutical Society of Ireland and members of the Pharmaceutical Society of Northern Ireland while these Societies remain associated with the Conference and pay annual contributions to the Conference in lieu of individual subscriptions from their members;

(*c*) elected members.

3. Elected members are those persons at home and abroad interested in subjects relative to the science of pharmacy who, not being members of the Pharmaceutical Society of Great Britain or one of the other Societies associated with the Conference, have been elected by the Executive.

* To take effect from September 7, 1963.

Proposals for membership by election must be made in the form approved by the Executive Committee and must be signed by three members of the Conference who recommend the candidate as a fit and proper person to be associated with the Conference.

4. The Officers of the Conference shall consist of a President, a Chairman, an Honorary Treasurer and two Honorary General Secretaries.
The President of the Pharmaceutical Society of Great Britain shall be the President of the Conference; the other officers of the Conference shall be nominated by the Executive Committee, and the nominations shall be subject to the approval of the annual meeting of the Conference.

5. The Executive Committee of the Conference shall consist of the Officers of the Conference, five past-Chairmen who have most recently filled the office of Chairman, together with three persons nominated by the Council of the Pharmaceutical Society of Great Britain and nine other members 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 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. Student members may not be members of the Executive Committee.

6. Of the nine other members of the Executive Committee for whom provision is made in Rule 5, three shall retire each year in accordance with seniority of service or otherwise as may be decided at the annual meeting of the Conference. Such retiring members shall be ineligible to serve again until after the lapse of one year. The vacancies so created shall be filled by election at the annual meeting of the Conference. Nominations shall be made by the Executive Committee, due regard being paid to the desirability of securing representation of the various branches of pharmaceutical practice and its associated scientific disciplines. Nominations may also be made by any five members of the Conference, in writing, to the Honorary General Secretaries at least 28 days before the commencement of the annual meeting. In the event of there being more nominees than vacancies a ballot shall be held in accordance with arrangements to be made by the Executive Committee. Any casual vacancy shall be filled by co-option.

7. The Chairman of the Conference shall *inter alia* give the inaugural address, preside over the scientific meetings and take the chair at the meetings of the Executive Committee.

8. Non-members of the Conference may attend the annual meeting of the Conference only as guests of members or at the invitation of the Executive Committee. They may not vote at any business session of the Conference. The Executive Committee may, however, decide that attendance at any particular session or function is to be restricted to members of the Conference. Student members of the Conference may not vote at any business session of the Conference.

9. Elected members shall pay an annual fee which will be decided from time to time by the Executive Committee. Subscriptions shall become due on January 1, and membership shall cease if subscriptions are not paid by June 1.

10. By arrangement with the Pharmaceutical Society of Great Britain, the transactions of the Conference are published in the *Journal of Pharmacy and Pharmacology*. Members of the Conference may obtain copies of the Journal on preferential terms.

H. G. ROLFE

Chairman, 1963

BRITISH PHARMACEUTICAL CONFERENCE

LONDON, 1963

CHAIRMAN: H. G. ROLFE

CHAIRMAN'S ADDRESS

THE BRITISH PHARMACEUTICAL CONFERENCE 1863–1963

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Introduction

It had always been customary, until 1922, for the President of the British Pharmaceutical Conference to deliver an address at the opening session, and thereafter it has been specified in the Constitution and Rules as one of the duties of the Chairman, as he was then named. For the one hundredth annual meeting it seemed appropriate for the Chairman to take the history of the Conference itself as the subject for his address, although the following extract from the President's address in 1913 gave pause for thought.

"It might have been fitting that on this, the fiftieth Annual Meeting of the Conference, the President should review the work of the Conference since its inception but I put that subject on one side because an adequate historical review would encroach unduly upon the time of this meeting and try your patience utterly!"

This account is based upon a study of the Proceedings of the Conference and of the Minutes of the Executive Committee, whose permission to do so is gratefully acknowledged. The following words, unless otherwise described, have the meanings indicated.

President: the President of the Conference; from 1923 this office has been held, *ex officio*, by the President of the Pharmaceutical Society of Great Britain.

Chairman: the Chairman of the Conference; prior to 1923 this officer was called the President of the Conference.

Executive: the Executive Committee of the Conference.

British Association: the British Association for the Advancement of Science.

Society: the Pharmaceutical Society of Great Britain.

Council: the Council of the Pharmaceutical Society of Great Britain.

Origin of the Conference

At a meeting (*P.J.*, 1852) of chemists and druggists of Bristol and Clifton in 1852, addressed by Jacob Bell, G. F. Schacht suggested that scientific meetings should be held annually—"which should circulate through the chief towns in the provinces"—as did the British Association. Another speaker said—"it had long been alleged as an objection that the benefits arising from the Society were confined to the Metropolis".

The Society had then been established for 11 years. During that period its main functions had been educational. It had its School of Pharmacy, was an examining body, and arranged meetings in London for the presentation of papers and the discussion of scientific matters. The above report and the letters to *The Pharmaceutical Journal*, mentioned below, show there was dissatisfaction among provincial members of the Society in that they were unable to participate as fully as they wished in assisting in the scientific advances of pharmacy; they also felt that they were not receiving as much benefit from the Society's activities as were the London members.

The first definitive proposal that a meeting should be called was made 11 years later by R. Reynolds of Leeds (*P.J.*, 1863a). He drew attention to the activities of the American Pharmaceutical Association, established 10 years previously, which like the British Association, then 32 years old, held annual meetings in different cities. Members accepted subjects for investigation and reported at the next meeting and committees were nominated to submit reports upon specific subjects, a procedure also adopted by the British Association. The published proceedings of the American Pharmaceutical Association included a valuable section which formed a Year-Book of home and foreign pharmacy.

He had previously consulted Schacht who, he said, had confirmed that his views about provincial meetings were unaltered and that he (Schacht) would object to the formation of a new organisation, since he regarded the existing means at the disposal of the Society as being fully capable of direction in the manner desired. Reynolds continued—"Our London brethren have chiefly maintained the scientific meetings of the Society for many years. The provincial constituency is four times as numerous and may fairly be called upon to exert a more systematic effort than at present. I would suggest that the coming meeting of the British Association at Newcastle-on-Tyne would afford the opportunity of testing upon a small scale the feeling towards such gatherings. Many pharmaceutists attend the meeting of the Association and it would not be difficult to devote a day to a Conference among them".

This proposal received an immediate response from H. B. Brady who offered on behalf of the Newcastle upon Tyne pharmaceutists to do all they could "to facilitate the objects of the meeting" (*P.J.*, 1863b).

Schacht (*P.J.*, 1863c) thanked Reynolds—"for the vigour and skill with which he advocates the old suggestion of mine and his own more enlarged ideas of scientific combination and pharmaceutical progress". It looks as though he must have had some unfortunate experience with a scientific communication he sent to a London meeting as he wrote—"Speaking from certain knowledge, I can state that several have for some time resolved to communicate nothing for discussion at the Society's meetings unless able to be present in person to defend their communications against the criticisms, candid and uncandid, to which they are sure to be subjected". His views of the London members of the Society are also shown by his statement—"Hence London members have done the largest share of the Society's work and have clearly enjoyed the largest share of the benefits; and in the meantime Scientific Pharmacy, to say the least of it, does not flourish".

The result was that 50 leading pharmaceutists, including several from London, invited (*P.J.*, 1863d) "their pharmaceutical brethren to unite in a conference"—to be held during the meeting of the British Association in Newcastle in September, 1863. The extracts below from this historic invitation show clearly their objectives and their desire to co-operate with the Society. After referring to the idea having been broached several years before, they stated—"We believe that the time has arrived for its realisation. Our body now includes some hundreds of members who

have received a professional training and the exertions which may fairly be demanded from these for the advancement of Pharmacy require co-operation for their most successful development. Our names will guarantee that the present proposal is not in the slightest degree antagonistic to the Pharmaceutical Society and we advisedly consider that the objects and interests of the Society would be promoted precisely to the extent that the Conference became successful and influential.

The objects of such Conference would be as follows.

To consult whether it is desirable to establish an Annual Conference on Pharmacy, either meeting at the place and time of the meeting of the British Association, or otherwise. If accepting such a proposal, to complete the organisation of an Executive; to recognise the various Provincial Chemists Associations and consider how best to promote their union; to discuss and allot for investigation, subjects in Pharmacy which demand inquiry, whether referring to new remedies, or processes or adulterations; to appoint committees for any allied object, etc.

The proposed plan of operations involves no costly outlay, no salaries, no publishing department; and viewed as an experiment, it is a perfectly safe one".

The Inaugural Meeting

At the inaugural conference (*P.J.*, 1863e) held at Newcastle upon Tyne on September 2, 1863, Henry Deane was elected chairman and "21 gentlemen" were present.

It was unanimously resolved—"That it is desirable that an Association be formed, to be called the British Pharmaceutical Conference, for the purpose of holding in the provinces an annual meeting of those engaged in pharmacy." Despite the wording of this resolution it should be noted that in the Constitution and Rules adopted by the meeting (see Appendix A) it was not stated that the annual meeting must be held in the provinces. It was emphasised that membership would not be limited to members of the Society as this would exclude—"professors, medical men who might be especially interested in the subject, professional chemists and the cultivators of medicinal herbs". Article II of the Constitution was included to—"prevent any suspicion that the Conference would be employed for purposes foreign to those put forward by the promoters".

Although after the election of the officers and Executive an invitation was accepted from the pharmacists of Bath, who had previously discussed the proposal with those of Bristol, to hold the first annual meeting in Bath in 1864 at the time of the meeting of the British Association, the newly established Conference promptly began to function. A committee of five was asked to report on "Adulterations"; a member showed a number of specimens of fraud or adulteration, including "Howards disulphate of quinine" sold at 6d. per oz. under the maker's price and found to be sulphate of quinidine, tartaric acid containing 12 per cent Rochelle salt, and opium containing 25 per cent of sand or earth.

The first paper ever presented to the Conference was read. The subject "Weights and Measures" has always been of vital interest to

pharmacists. The situation does not appear to have greatly changed judging from the following extract from the discussion—"the adoption of the decimal system had become a practical question for the legislature. Its principle had been sanctioned by the vote of the House of Commons and doubtless a Bill upon the subject would again be introduced next session. The decimal system therefore was likely to be attained. . . ." It certainly seems to be in sight a century later!

In the evening the first social event of the Conference to "promote friendly reunion" among its members, was a supper at which not less than eight toasts were drunk! As an illustration of the friendly feelings of the members towards the Pharmaceutical Society, one speaker—"claimed for the new organisation the best wishes and anything more of 'the old lady of Bloomsbury Square' ".

Changes in the Constitution and Rules

1864–1921

The first change in the Rules was made in 1865 when it was decided that those who had filled the office of President should be created Vice-President and be members of the Executive Committee. As a result there were two classes of Vice-Presidents, the four (increased to not exceeding six in 1902) elected each year, and the past-Presidents. The subscription was raised to 7s. 6d. in 1873 to cover the increased cost of the *Year-Book of Pharmacy*, a copy of which was sent to every member post-free, and again to 10s. 6d. in 1919.

In 1907, the Executive considered, but did not accept, a suggestion by a member that the constitution should be revised so that one of the objects should be—"for affording opportunities for the consideration of subjects germane to the calling of pharmacists"—he also wanted local associations to instruct delegates to attend the Conference for the discussion of—"political and ethical subjects". However, a paper presented to the 1909 Conference entitled "Should the dispensing of medical prescriptions be exclusively confined to pharmacists?" created much interest and led to the view being accepted that the discussion of subjects of a more general nature should be permitted. The words "advancement of Pharmacy" in Art. I, 1, were changed to "advancement of the Science and Practice of Pharmacy" and Practice Sessions began in 1911.

In 1913, the Executive appointed a sub-committee to consider how the usefulness of the Conference could be increased and it had discussions with the President and Secretary of the Society regarding the possibility of the Conference joining forces with the Society. In 1915, it recommended the Executive to have further informal discussions with representatives of the Society on "the advisability of an amalgamation of the Conference and the Pharmaceutical Society" but owing to the War no action was taken.

The changes made to the Constitution and Rules during the first 58 years were only minor adjustments to meet changing circumstances, and reflect highly upon the sound judgment and prescience of the Conference founders.

1922–1962

In 1922 the Conference passed through the most difficult period in its history. At the meeting of the Executive held on January 18, a letter from the Secretary of the Society, Sir William Glyn-Jones, was considered. It referred to the recommendation to Council by its Organisation Committee, which had been adopted.

"The Society shall organise at least once a year, a National Conference, not necessarily meeting in the same place each year, and the Conference shall consist of delegates officially appointed to represent Branches of the Society. The Conference shall deal with the Science and Practice of Pharmacy, and will be concerned with the general advancement of the objects of the Pharmaceutical Society".

The letter stated that this development must—"have a bearing on the work of the British Pharmaceutical Conference"—and suggested that the matter be discussed between representatives of the Executive and the Council. A sub-committee of the Executive was appointed accordingly for this purpose.

This move by the Council appears to have been completely unexpected. There is no indication in the minutes of the Executive that any prior discussion had taken place. This is the more surprising in that during the period 1913–1915, as mentioned above, the Executive considered amalgamation with the Society. On February 1, the Executive sub-committee met representatives of the Council and the President of the Society made a statement explaining the Council's proposals. The main points were as follows.

As a result of the decision of the Jenkin case the Society was relieved from its activities connected with trading and was now free to devote more attention to the first of its chartered objects—"the purpose of advancing Chemistry and Pharmacy". The Council had decided to form Local Branches of the Society and to hold a National Conference which would deal with the domestic affairs of the Society, as well as the subjects dealt with by the British Pharmaceutical Conference, although some subjects previously discussed at the Practice Section of the latter on trading matters would be barred.

There would in future be two Conferences unless those who were interested as members of the British Pharmaceutical Conference were satisfied that the efforts of all should be united in one Conference under the Society's auspices. In that event the name "The British Pharmaceutical Conference" would be retained and he hoped the Executive would accept this course "in view of the altered circumstances and of the decision of the Council to so greatly increase the Society's activities for the attainment of the very objects for which the British Pharmaceutical Conference exists". The Council "gratefully acknowledge the splendid work that has been done by members and non-members of the Society" in the Conference in the past, and were anxious to secure their active co-operation in the future. Although the Council would have the power of veto, he suggested that the Executive, whose members consisted of both members and non-members of Council, could form an Expert Committee

to which Council would delegate the duty of running the Conference. If the Executive agreed, he was authorised to state that the one Conference, organised by the Society, could be run by the Executive as a Council Committee, so ensuring continuity.

The Council would then make persons of distinction in the realms of Chemistry and Pharmacy, who were helping the Conference, honorary members of the Society and it would be possible to make arrangements for others, who were not pharmacists, to attend and assist in its work. "Whilst the President of the Society would be President of the Conference, Chairmen of Sections and a person to read the Inaugural Address could be appointed on the nomination of the Conference Committee". The Society would take over responsibility for the production and publication of the *Year-Book of Pharmacy*, the terms of supply to members and others to be decided later.

Finally, if these proposals were "approved in principle" by the Executive he proposed that a small joint committee of Council and Executive should draw up a detailed scheme for presentation to the 1922 Conference, which was to be held at Nottingham.

If the Council had wished to arouse opposition to a merger they could hardly have acted more effectively and one can imagine the feelings of the members of the Executive, in particular at the general tone of the proposals involving decisions already taken and not apparently subject to negotiation. Incidentally, the President of the Society and five other members of its Council, were members of the Executive at the time!

On February 23, the Executive decided "that they would not be warranted to negotiate for the discontinuance of the Conference as at present constituted". They could not accept the Council's proposals "in principle" and therefore it was "not feasible" to nominate members to the proposed joint committee. They offered facilities on one afternoon, however, at the 1922 Conference meeting for the Society to present their scheme to the Conference members.

The Secretary of the Society replied on March 16, noting the Executive's view that "the general body of members must have an opportunity of considering the situation, and of expressing their views before a concrete scheme is presented to them for adoption" but he did not refer to the offer of time at the forthcoming Conference for the Society to present their proposals.

On May 3, in reply to a further enquiry about the offer of time, he informed the Executive that as they could not accept the proposals "in principle", it would be best for them to explain the position to their members. The President of the Society proposed to explain the Council's scheme at the Society's Annual General Meeting.

Thus the Executive's refusal to be forced to negotiate led to a situation which was very unsatisfactory from their point of view. Looking back, the policy adopted by the Council at the beginning of this unhappy episode of—"amalgamate or else, the decision has been taken"—instead of attempting to achieve their purpose by negotiation with the officials of a body with whom relations had been harmonious for nearly 60 years was

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most unfortunate. One cannot say the objective was undesirable but the tactics employed were certainly unwise.

Having reached this apparent deadlock, more moderate counsels prevailed. On the one hand the Executive decided that although it could not, without the sanction of the Conference, proceed with any negotiations with the Society, it was their duty to submit to the Conference the correspondence and the Society's proposals contained in its President's statement to them with an indication of the course of action they considered it would be wise for the Conference to take. On the other hand the President of the Society indicated he was prepared to recommend modifications to the original proposals of the Council.

Further discussions took place and on May 24 the Executive reconsidered the whole matter including the modifications of the policy set out in his original statement to be recommended by the President of the Society.

They stressed the importance they attached to the voluntary effort and spirit which had supported the Conference from its beginning. The policy followed had ensured the co-operation of all sections connected with pharmacy and no one interested in its welfare could take exception to the Society taking an active part in the work the Conference was doing but the decision of the Council to hold annually a National Conference had to be taken as final and two such Conferences were clearly undesirable.

The Executive considered the following to be of great importance to the Conference:

(*a*) that persons not qualified as pharmacists were able to work for it, to the advantage of Pharmacy;

(*b*) the annual presidential address, and

(*c*) that the work between the annual meetings and arrangements for papers to be read were in the hands of those selected by members of the Conference.

The main points of the recommendations resulting from the discussions were as follows:

1. The name "The British Pharmaceutical Conference" to be continued.

2. Those specially qualified to assist in the scientific work of the Conference, who were not pharmacists, to be elected honorary members of the Society. This group would include all such persons who were past-Presidents of the Conference.

3. The election, on the nomination of the Executive, as corresponding members of the Conference, of other persons residing either at home or abroad, who were not qualified for membership of the Society. Such corresponding members would be eligible for election as officers of the Conference.

4. A Chairman of the Conference to be elected each year. He would give the inaugural address and preside over the meetings at which science papers were read and at meetings of the Executive.

5. A Conference Executive to be elected at each Conference, at least one-third of the members, other than the officials, to be members of the Council. The Council would then "appoint as the Executive of the Conference the members so selected, and they will delegate the work of directing the whole of the affairs of the Conference to such Executive".

6. The Society would undertake the production of the *Year-Book of Pharmacy* in which they might include part of the matter published in their Calendar. Members and student-associates of the Society would automatically become members of the Conference and their subscription to the Society would include the right to receive, on application, a copy of the Year-Book. Corresponding members of the Conference would continue to pay the subscription of 10s. 6d. to the Conference which would entitle them to receive a copy of the Year-Book.

7. Members of the Society, through their local branches, would elect official delegates to the Conference and it was expected there would probably be about 150 such delegates.

8. When Society matters were under discussion it might be that the Council would require the opinions of delegates only, in which case, only the latter would be able to vote.

The Executive "decided that it should be reported to the members at Nottingham that after weighing the considerations for and against the continuance of the Conference as a separate function additional to the Society's Conferences, they have with reluctance arrived at the opinion that on balance, an acceptance of the Society's proposals is desirable provided the modifications supported by the President of the Society at the joint conferences and at meetings of the Executive are embodied".

This resolution was the unanimous view of those present at the Executive meeting, but other members who were not present adhered to the original decision of the Executive and considered members of the Conference should have an opportunity of considering the situation untrammelled by such resolution.

Full details of the correspondence with the Society and of the discussions which had taken place were circulated to every member of the Conference before the Nottingham meeting (*Yearb. Pharm.*, 1922). At that meeting a lively and prolonged discussion took place.

The main arguments of those who were opposed to the proposals were as follows:

(*a*) The Conference had been free and independent as, unlike the Society, they were an unofficial body and they could express any opinion "whether irresponsible or otherwise".

(*b*) Membership of the Conference was broader than that of the Society, both in the United Kingdom and overseas.

(c) Before the Council came to a definite and final decision the Conference should have been consulted.

Eventually the report was adopted by a large majority and the new Executive was authorised to make the consequential alterations to the Constitution and Rules. The new circumstances required that these be rewritten and they were published in the *Year-Book of Pharmacy* for 1922. They were approved by the Council in November, 1922, and are set out in Appendix B.

Although not permitted by the Rules, in 1923 the President of the Pharmaceutical Society of Ireland and the Chairman of the North British Branch of the Pharmaceutical Society of Great Britain, and in 1926 the President of the Pharmaceutical Society of Northern Ireland, were added as *ex officio* members of the Executive. These additions were welcomed. No one drew attention to the irregularity but the position was eventually corrected in 1931 when Rule 4 was amended and, in addition, of the six elected members, the two with the longest period of continuous service became ineligible for re-election for one year. At the same time the opportunity was taken to include the Chairman of the Local Committee as an *ex officio* member.

In 1927, it was realised that the Constitution was so phrased that members of the Pharmaceutical Society of Northern Ireland could only become associated with the Conference as corresponding members. This position was unsatisfactory and it was considered possible that other Societies in the Commonwealth might wish their members to become full members without the necessity of individual nomination as corresponding members. The matter was discussed with the Council and in 1934 Rule 2 was rewritten to include the old Rule 3, and the other rules were amended where consequential changes were necessary. The Pharmaceutical Society of Northern Ireland agreed to pay an annual subscription of £25 for its members.

The revised Rule 2 read:

The Conference shall consist of:

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

(*b*) Members of 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.

Consequential changes were necessary after the publication in 1928 of the *Quarterly Journal of Pharmacy*, incorporating the *Year-Book of*

Pharmacy, and again when it became the monthly *Journal of Pharmacy and Pharmacology* in 1949. The Editor of the *Journal of Pharmacy and Pharmacology* became an *ex officio* member of the Executive in 1956.

Last year the revised Constitution and Rules, set out in Appendix C, were accepted and will take effect after the 1963 Conference. A number of changes of substance have been made and the whole has been rewritten in line with modern conditions and to make the meaning as clear as possible.

No approaches had been received from Commonwealth Societies to implement the change in Rule 2 made in 1934, and it was therefore decided to restrict corporate membership, additional to the Pharmaceutical Society of Great Britain, to the Pharmaceutical Societies of Ireland and of Northern Ireland. Elected members must now be nominated by three members of the Conference—the previous arrangement of one nominator dated from the very early days of the Conference and was open to abuse. Three changes have been made in the membership of the Executive Committee. The number of past-Chairmen has been restricted to five, the three persons nominated by the Council need not necessarily be members of that Council, and the number of elected members has been increased from six to nine, of whom three shall retire each year.

It is stipulated that the Executive, in making nominations, shall pay due regard to representation on it of the various branches of pharmaceutical practice and its associated scientific disciplines. Further, any nominations, other than by the Executive, must be made by five members of the Conference, instead of one as hitherto. It is laid down that non-members of the Conference may attend the annual meeting only as guests.

The subscription paid by the elected members will not, in future, entitle them to receive the *Journal of Pharmacy and Pharmacology* without further payment but they will be entitled to obtain it on the same preferential terms as other members of the Conference.

Membership and Finance

At the end of 1864 the number of members of the Conference was some 200. This figure steadily increased during the succeeding years to 700 and as the result of a circular sent to pharmacists in England in 1870 it rose to about 1,500. From 1870 members were provided with a copy of the *Year-Book of Pharmacy* and this encouraged those who were unable to attend the annual meetings to become members, as no payment additional to the annual subscription was demanded. The membership had reached about 2,750 by 1875 and for the next 12 to 15 years it fluctuated between 2,000 and 2,600. Thereafter, the number gradually fell, but except for a short period during the first World War, it always exceeded 1,000.

The method adopted to obtain members was to send periodic circulars to pharmacists and their assistants and to newly registered chemists and druggists. Heads of schools of pharmacy and the secretaries of local pharmaceutical associations were also asked to help.

In 1883, a number of Honorary Colonial Secretaries were appointed and this led within two or three years to nearly 400 additional members. In 1899, 39 Local Corresponding Secretaries were appointed and their number was increased to 80 in 1905. Their duties were as follows:

(*a*) To bring under the notice of pharmacists, principals and their assistants in their districts, who were unassociated with the Conference the advantage of membership with it and by personal effort to try and induce them to join.

(*b*) To assist in stimulating research by asking pharmacists who have the time, ability and disposition, to contribute from time to time, a paper or useful note to its annual meetings.

(c) To endeavour to induce defaulters to continue their membership.

(*d*) To take generally a watchful and sympathetic interest in the affairs of the Conference.

To render these services voluntarily at times convenient to themselves and as opportunity offers.

Great difficulty was experienced in collecting subscriptions, for example, in 1877, of some 2,550 members, nearly 500 had not paid; in 1899 about 25 per cent were in arrears and in 1912, about one-third. Periodically, those greatly in arrears were struck off the roll of members which was published in the Year-Book.

Occasionally, members had to be disciplined for other reasons. In 1875, a member was asked to resign, or be expelled, as he had been imprisoned for stealing from his employer. In 1879 and 1885, the Secretaries had to deal with members who had broken Article II of the Constitution in that they made improper use of their membership, one describing himself as an M.B.P.C. In 1895, a member was struck off for the same reason.

One other class of member must be mentioned, namely honorary foreign members. A minute of the Executive for 1868 reads—"It is expedient to include in the list of members of the Conference the names of gentlemen, not resident in Great Britain but identified with the progress of pharmacy or conspicuous for their attainments in sciences allied thereto, who have contributed to its usefulness or in other ways shown themselves interested in its proceedings". A short list of honorary foreign members appeared in the Proceedings for many years.

Income

The income of the Conference until 1922 was almost entirely derived from subscriptions but gifts were also received from various sources. For example, in 1897 and 1898, the local committees of Liverpool and Glasgow each gave £20 from the balances remaining after their Conferences. Other sums were given by Dundee, in 1903; London, in 1919 and Liverpool, again in 1920. In 1953, London passed to the Executive £250, to be loaned to a Branch receiving the Conference until it could raise its own funds, or used at the Executive's discretion. Last year, Liverpool revived their old custom and gave £50 to the Conference funds.

It would be of considerable service to the Executive in the future if all hosts of the Conference, when they find that they have a surplus, will make a similar gift.

Bell and Hills Fund

This fund began in 1870 when Thomas Hyde Hills gave 50 guineas to the Conference, 25 guineas in memory of Jacob Bell and 25 guineas on his own behalf. He suggested (*Yearb. Pharm.*, 1870) that 10 guineas should be used for the purchase of books for the chemists' library in the towns visited by the Conference each year, but made no other stipulation. Two years later he added four £50 Russian bonds which provided interest of £10 per annum. In addition to providing books this money was to be used (*Yearb. Pharm.*, 1872) at the discretion of the Executive, to make grants in aid of original research or in any other way desired for the advancement of pharmaceutical science. Several research grants were made. One bond was sold in 1874 and he provided a further £50 Russian bond in 1876. A year or so later, the Executive sold the bonds and re-invested the money together with sufficient additional cash to purchase £350 Consolidated Stock which at that time was paying 3 per cent interest to give an annual income of £10 10s. Henceforward the capital remained untouched and the interest was used to provide books for the local associations of the Conference towns which could use them to advantage. In 1889, the £350 of 3 per cent Consols was converted to £360 of 2¾ per cent Consols, the income thus being reduced to £9 18s. In 1903, the rate of interest was further reduced, this time to 2½ per cent, where it has remained ever since. The gift of books lapsed during both World Wars and since 1952, a memento, usually in the form of a gavel, has been presented to the host branch of the Society as branches no longer had libraries. £60 from the Bell and Hills Fund was paid in 1946 to the Corfield Memorial Fund.

In 1918, an anonymous donation of £1,250 (nominal) Consols was received, the interest to be used for the benefit of pharmacy in any way the Executive considered appropriate. After his death it became known that the donor was Alderman Clayton of Birmingham.

From 1864 until just before the end of the century the finances of the Conference showed a credit balance which reached a maximum of over £500 in 1880 and 1881 but from 1899 to 1912 the Conference was in debt. A surplus was again built up during the first World War but this rapidly disappeared afterwards and there was a deficit when the association with the Society took place in 1922.

In 1951, the Pharmaceutical Society of Northern Ireland increased its annual contribution to £50. Since 1955 the Pharmaceutical Society of Ireland has subscribed £25 annually which has been increased to £50 this year.

Expenditure

The expenses of the Conference fell into three main groups—grants for research, administrative and publications. In the early years, two payments

of £25 each were made to local Committees and in 1951 a payment from the general fund made good a deficit in the local Committee accounts.

Grants for research, about £5 or £10 for the purchase of materials or equipment, were made particularly to those undertaking research into subjects on the Research List; it was a frequent cause for regret that more requests were not received.

Administrative costs included not only postage and printing but also, from 1870 until after the First World War, the salary and expenses of a part-time assistant secretary and sub-treasurer which varied over the period from £25 to £110 per annum.

The major expense was the publication of the Year-Book. The first Editor was paid £100, later increased to £150 per annum. The cost of the publication was in part defrayed by income from advertisements. In 1899 reference is made to the need for more income from this source and in 1905 the Treasurer complained that advertising revenue had been adversely affected by appeals from local committees to advertisers for contributions to the local expenses of the Conference.

Substantial expenditure was incurred by the publication on three occasions of a General Index to the Year-Book, those of 1870–1885, 1886–1903 and 1904–1927. The first was published at a time when the membership subscription was such that there was an adequate balance in the general fund. The second did not sell to the extent envisaged. By 1906 only 206 of 500 copies printed had been sold and there was a deficit of some £200 in connection with this item alone. This was dealt with by making a special appeal and £164 was subscribed by 50 members. With this help and some improvement in the general financial position the deficit was eliminated by 1911. The financing of the Year-Book was taken over by the Society in 1922 but a little later there was pressure for the third General Index. At first the Council considered the project too costly but in 1928 they agreed to pay half any deficit on it and D. Lloyd Howard made a gift of £100 towards the cost. The 1904–1927 General Index cost £722 and in 1930 the receipts from sales together with the supply of unsold books and the copyright were passed to the Society together with Lloyd Howard's £100 and the total balance in the Conference general fund of £258 0s. 11d., the excess cost being made good by the Society.

The financial position was changed with the association with the Society in 1922. The investments in Consols from the Bell and Hills Fund and the Clayton gift were retained by the Conference and the Society met the deficit in the accounts for the year 1922. In 1931, the Society, not unreasonably, allocated the subscriptions from corresponding members (later called elected members) to their account for the *Quarterly Journal of Pharmacy and Pharmacology* and the subscription of £25 paid by the Pharmaceutical Society of Northern Ireland from 1934 was also passed to the same account.

By 1951 the Conference had again accumulated a reasonable credit balance in the general fund and a Chairman's badge and replicas for past-Chairmen were purchased. In recent years the Conference has been able

to provide from its own resources for the expenses of invited speakers and lecturers to its meetings and for certain administrative costs, although in 1958 it was discovered that its income was not exempt from income tax and a back payment of £69 15s. 6d. was agreed with the authorities. In 1962 it was apparent that a very small number of authors of science papers in academic institutions might be unable to present their paper in person to the Conference for financial reasons and arrangements have been made to provide assistance to such authors in future.

RELATIONS WITH THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

Although the Conference had its origin in what some considered to be certain shortcomings of the Society, nevertheless, the founders did everything possible to ensure that the Conference was complementary to the Society and in no way in competition with it and relations between the two bodies were cordial from the outset. Indeed the President of the Society, G. W. Sandford, wrote (*P.J.*, 1868) in 1868.

"I have always regarded the Conference as an admirable offshoot of the Pharmaceutical Society, perhaps I might more properly call it an 'outburst' for I do not claim any credit for the Society in establishing the Conference, although I do consider the relationship between them as very intimate".

In the same year a resolution was passed at the annual meeting of the Conference thanking the President of the Society for his efforts in connection with the 1868 Pharmacy Act and requesting that recognition should be made of his services to pharmacy. As a result some £500 was raised, part being used for a presentation of plate and the remainder for his portrait to be hung in 17 Bloomsbury Square.

One of the objectives of the original Constitution—"to form a bond of union among the various associations established for the advancement of Pharmacy"—was implemented by inviting attendance at Conferences of delegates from the Society, the North British Branch of the Society, the Pharmaceutical Society of Ireland, as well as the local associations and by 1888 over 30 such local bodies named their delegates, although many of them did not attend. As a result of the Conference visiting Dundee in 1868 a local pharmaceutical association was formed in that city (*P.J.*, 1868).

A speaker at the 1872 Conference reflected no doubt the views of a number of pharmacists at the time of the inauguration of the Conference—"It is not so many years since the Conference was first projected. Certain sage individuals shook their heads very ominously and said that the Conference would possibly, nay very probably, do a deal of injury to the Pharmaceutical Society".

The President expressed the general view when he said "There is no difference in the objects of the two institutions; they are and must be, perfectly harmonious and complementary to each other. The particular methods open to them to attain the same end—*the advancement of pharmacy*—differ considerably, but only as different roads to one goal. Without the Pharmaceutical Society the Conference could never have

been; with the establishment of the Conference the best days of the Society dawned. The success of each must be the chief delight of the other".

The atmosphere changed abruptly in 1922 and the events which then took place have been described in the section entitled "Changes in the Constitution and Rules". The 1923 Constitution and Rules dealt with the more important aspects of the new arrangements but there was a lack of clarity and of understanding on a number of points. Some difficulties were inevitable from such a "shot-gun marriage" and no doubt many individuals on both sides had varying ideas as to what was intended. The powers of the Executive were subjected to pressure; for example, the President in 1923 stated at an Executive meeting that the place of meeting for 1924 should be referred to the Organisation Committee of the Council but no action appeared to have been taken. The general view of the relationship of Conference and Society was probably expressed in 1934 by a member of Council who remarked that—"the amalgamation . . . seemed to have been fairly successful".

There were two major causes of friction. In the 1923 Constitution the President of the Society became *ex officio* a member of the Conference Executive and President of the Conference but nothing was done to clarify what, if any, were the duties of the President of the Conference; in fact, the title was not used for many years.

The delicacy of relations between President and Chairman was voiced by the Chairman in 1924 when he said—"the position of the Chairman is not without difficulty—or might not be, unless the spirit of co-operation is exerted between the President of the Society and the Chairman of the Conference". The situation was resolved in 1952 when it was made clear that the President of the Society attended the Conference in his capacity as President of the latter body. It was agreed that the President would take the chair at the opening of the Conference, reply to the address of welcome, call upon the Chairman to deliver his address and at that point hand over the responsibility for the further conduct of the annual meeting to the latter. The duties of the two persons at the social functions were also amicably agreed.

The second difficulty was in connection with Branch delegates. One of the arguments for the association of the Conference with the Society had been that the latter would send delegates from their branches to the Conference. However, the arrangements for the meetings of branch delegates and for deciding the subjects to be discussed were handled directly by the Society and quite separately from other Conference matters from 1923 onwards. This resulted in a dichotomy of the Conference and a gradually diminishing attendance of delegates at the whole Conference, which was not remedied until 1956.

It was in 1953 that a suggestion was first made to the Executive by the Council that they (the Council) might be willing to discontinue their Branch Representatives Meeting held during Conference week and to send delegates from their branches to the Conference as a whole. The Executive decided that if this meeting was discontinued they—"would

make adequate provision within the Conference programme for discussion of matters of a professional nature, one day or two half-days being provided for sessions devoted to the discussion of such subjects. A separate Chairman for the new session would be appointed by the Executive and it is anticipated that normally they will request the President of the Conference to undertake this duty".

The Council published a statement (*P.J.*, 1955) explaining their reasons for the change and the new arrangements were implemented at the 1956 Conference. The new sessions were called "Professional Sessions".

The improvement in the relations between the two bodies after the last War was reflected also in the financial support the Council provided to enable the Executive to assist young pharmacists, particularly those who had completed their National Service, to attend the Conference. During the years 1948 to 1955, 233 young pharmacists were provided with books of Conference tickets and free travel to the Conference. In 1956, it was decided that this help was no longer necessary as branches of the Society could help their younger members themselves by nominating them as their delegates to the Conference.

It is very gratifying that the relationship of the two bodies is now back where it was in the early days of the Conference and the wounds and bruises suffered in 1922 are completely healed.

Conference Publications

The four important publications of the Conference have been as follows: The Proceedings (Transactions) of the Conference. The Research List. *The Year-Book of Pharmacy. The Unofficial Formulary.*

The Proceedings (Transactions) of the Conference

The Executive decided in 1864 that the papers read at the Conference that year and the discussion of them reported in *The Pharmaceutical Journal*, together with the statement of the objectives of the Conference, the list of officers, a table of contents and a list of members, and entitled "Proceedings of the British Pharmaceutical Conference, Bath Meeting, 1864", should be printed and a copy sent, post-free, to each member and to "eminent scientific men" and learned Societies in England, America, Germany and France. This procedure was followed until 1870 when the Proceedings were no longer published separately but were included in the *Year-Book of Pharmacy.*

The Research List

The objectives of the Research List were not only to stimulate research but also to attempt to allocate the investigation of problems to individuals. A copy was sent to each member after their election and a new list after each annual meeting.

The first list, published in 1863, comprised 24 "subjects for research" and 16 "subjects relating to adulteration". The former included the preparation of syrup of senna, and ergot—"what is its active principle and what is the best preparation for its administration?" The latter

covered—"impurities and faults of manufacturing"—of a number of substances including potassium iodide and bismuth carbonate, the amount of alkaloid in various specimens of citrate of iron and quinine and "the composition of the bottled mineral waters of commerce".

The list was revised regularly. In 1871 it was described as the "Blue List"—presumably because it was printed on blue paper marked—"for private circulation only"—and the names of persons who had accepted subjects were included. By 1873 it comprised some 200 subjects and in 1876 the Executive stated—"In 13 years of about 200 subjects proposed, about 100 had been investigated resulting in papers forming about one-third of the 300 papers read at the Conference".

By 1878 there were subjects in the list which were considered to be outside the interests of the Conference, and the Blue List for that year was reduced to include only subjects possessing more or less special pharmaceutical interest so as to encourage the presentation of papers bearing closely on pharmacy.

Annual revision continued and it comprised 41 subjects in 1903. After the first World War, it was completely revised in 1922 but thereafter remained unaltered until 1932 when a new list was issued comprising 70 subjects; it was included in the Annual Report of the Executive for that year. This list was revised annually until 1939.

After the War the Executive reviewed the Research List in all its aspects and found that (1) very few papers presented to the Conference in the 1930's had been inspired by the list, (2) a list could possibly be misleading in that it might give the impression of being comprehensive, (3) research workers in industry and academic institutions considered a list to be of little value and (4) other workers who might wish to undertake research required more assistance than could be provided by a mere list of possible subjects for investigation.

In 1948, the Executive suggested that a Pharmaceutical Research Council should be set up to co-ordinate and stimulate pharmaceutical research work. The proposal envisaged a council consisting of representatives of a wide group of official bodies with a permanent officer of the Society devoting a large part of his time to its work. The scheme was probably over-ambitious and impracticable at the time. However, some of the objectives in mind at that time such as helping in co-ordinating research work and giving advice to research workers have been realised by the formation of the Society's Department of Pharmaceutical Sciences in 1959.

The Year-Book of Pharmacy

It was in 1868 that the publication of a Year-Book, or Annual Report on the progress of Pharmacy, by the Conference was agreed as soon as funds were adequate. The proposal was stimulated by a similar publication issued by the American Pharmaceutical Association. A publication committee and editor were appointed and the first number of the *Year-Book of Pharmacy* was issued in January, 1871. Two thousand copies were distributed.

The Year-Book contained abstracts of papers on materia medica, of both vegetable and animal origin, pharmaceutical chemistry, organic, inorganic and analytical, pharmacy, including preparations, processes and apparatus, together with notes and formulae, book reviews and the Proceedings of the Conference. The last named was the responsibility of the Conference Secretaries and not of the Editor.

It became customary for the editor of the Year-Book to include as an introduction a review of the developments in pharmacy and related sciences during the previous year. With minor modifications to reduce the cost of the book, it was published in a similar form until 1912 when two sub-editors were appointed to be responsible for sections on new remedies, and dispensing notes, respectively.

In 1922, responsibility for the publication passed to the Society with a joint advisory committee of three members of the Council and three members of the Executive. It was continued under the old name and part of the Calendar of the Society was included.

In 1928, the *Year-Book of Pharmacy* was incorporated in a quarterly journal—the *Quarterly Journal of Pharmacy and Allied Sciences*—the title being changed the following year to the *Quarterly Journal of Pharmacy and Pharmacology*. An editorial committee was constituted including three representatives of the Executive. The reason the Society made this change was to provide facilities for the publication of original scientific work and it was appropriate to do so at that time as the Society's Pharmacological Laboratories had been established in 1926. The only original research papers appearing in the Year-Book had been those presented at the annual meeting of the Conference.

In 1949, the *Quarterly Journal* became the monthly *Journal of Pharmacy and Pharmacology*. One result of this change was that the papers presented to the Conference were published over several monthly numbers of the Journal. For some years this was a considerable advantage to the Journal; it might have been difficult otherwise to provide 12 monthly numbers. Gradually, however, the number of papers from other sources increased so that the Conference Proceedings are now published in one (thirteenth) number as a supplement to the Journal. In the new Constitution and Rules the Society has undertaken to continue the publication of the Conference Proceedings in the *Journal of Pharmacy and Pharmacology* thus proving unfounded the fears of some that Conference papers might eventually be excluded. No one will dispute the necessity for papers presented at the Conference to be published in a reputable scientific journal; if this were not so, many research workers would be unwilling to present their papers to the Conference.

The Unofficial Formulary

In a paper presented to the 1880 Conference entitled "New and Unofficial Pharmaceutical Preparations", Charles Symes pointed out the need for some standing authority to sanction formulae for products not described in the Pharmacopoeias. He suggested that this might be done

as a separate section of the British Pharmacopoeia or by some lesser body but with more authority than a notice in journals.

In 1886, the President suggested that the Conference should publish formulae in its Proceedings for products such as mist. magnesiae et bismuthi comp. (Jones) as the "Unofficial Formulary of the British Pharmaceutical Conference". During the discussion of this proposal the view was expressed that the Conference rather than the Society was the appropriate body to publish such a formulary because the latter was fully occupied with educational matters and it would be embarrassed in its relations with the Pharmacopoeial Authorities if it did so. The underlying reasons are revealed in the following resolution which was passed.

"That in order to secure greater uniformity in composition and strength in non-official remedies and also to enable the medical profession to prescribe them with definite knowledge of those qualities and without indicating any particular maker, the Conference undertakes the preparation of a formulary of non-official remedies."

A Committee was formed to undertake the work and £25 placed at their disposal for expenses. The first edition of the Unofficial Formulary (containing 37 monographs) was published in 1887. It was printed in the Year-Book and copies were also sold at 6d. each in paper covers or 1s. each interleaved in cloth covers. By 1888, 2,250 copies had been sold.

In the introduction, the following sentence appeared. "In order to indicate clearly that the formulæ of the Unofficial Formulary are intended, it is suggested to the prescriber to add the letters B.P.C. (British Pharmaceutical Conference)." So the Conference began educating medical men to put the letters "B.P.C." on their prescriptions!

The Unofficial Formulary Committee was reappointed each year at the annual meeting of the Conference and in 1888 was given power to publish formulae provisionally, without the full approval of the Conference, provided seven of the 10 members of the Committee agreed, in order to provide formulae for preparations of new drugs. An Addendum to the first edition was published in 1889 and new editions in 1891, 1894 and 1901. In the 1891 Addendum to the B.P., seven Unofficial Formulary formulae were included and 18 in the B.P. 1898. The title was changed in the fourth edition to the B.P.C. Formulary.

In 1903, to meet difficulties which had arisen in connection with the Medicine Stamps Duty Acts and the interpretation of the phrase—"known, admitted and approved remedies"—the Council decided to publish "A Compendium of Medicines". Discussions took place between representatives of the Executive and Council regarding the position of the B.P.C. Formulary. In April, 1904, the Executive agreed in principle to dispose of the Formulary and the unpublished work of the Formulary Committee but they had no power to do so without the consent of a general meeting of the Conference. A special General Meeting was therefore held on May 17 to consider the "suggested remuneration for formulae and goodwill" but it was called by notice in the trade journals and several members protested that the proceedings were out of order as the meeting had not been called by individual notice to each member.

However, the proposal was accepted but details were left to be settled later by the Presidents of the Conference and of the Society who agreed that the Society would pay the Conference 70 guineas as compensation. At the annual meeting in 1904, there was an animated and, at times, bitter discussion, but the sale was finally approved by a majority of 41 to 19.

In the introduction to the 1907 British Pharmaceutical Codex (the title eventually used for the "Compendium of Medicines") the British Pharmaceutical Conference Formulary was mentioned as being one of the publications taken into account in compiling the book.

Places of Meetings

At no time in its history has the Conference had any difficulty in finding a town in which to meet. From 1864 to 1873 it received invitations from pharmacists of the towns at which the British Association was meeting, in some cases from others as well, but the British Association town was always chosen. The Conference meeting lasted three or four days and partly overlapped with that of the British Association.

In 1874, however, the British Association was to meet in Belfast and at that time feeling in Irish pharmacy was greatly stirred by prospective legislation. The position was confused but the following extract from an Executive minute is illuminating—". . . it appeared that in Ireland there were apothecaries practising medicine and pharmacy, apothecaries practising pharmacy but not medicine, and 'druggists' practising neither medicine nor pharmacy". It was considered important that being a neutral body the Conference should not show sympathy with any particular section and it was decided not to visit Belfast at that time. London was suggested and the President of the Society, Thomas Hyde Hills, who was also an elected Vice-President of the Conference, wrote to the President on the instructions of the Council offering the Society's house for the 1874 meeting should the Executive wish to meet in London for the first time in that year. Although some doubt was expressed about the wisdom of holding a Conference in London because the original proposals envisaged meetings in the provinces, the invitation was gladly accepted.

In 1879 and 1880, the Executive had further thoughts on the wisdom of continuing to hold the annual meeting with the British Association in August or September but decided to make no change for the time being. The advantages were that it was convenient to many, especially those who also attended the British Association meetings, it helped to work up local pharmaceutical enthusiasm when the whole town was about to entertain that body, it was a convenient time of year for teachers who attended and presented papers and the numbers were larger than they would be at any other time. The disadvantages were the difficulties of the local officials who were often helping to organise both meetings, the excursion clashed with British Association meetings and there was difficulty in obtaining hotel accommodation.

In 1884, the British Association visited Montreal and the Conference

visited Hastings, but as the number attending was less than 100 they reverted to the old practice of going with the British Association in 1885.

In 1892, a resolution was proposed at the annual meeting by a Belfast representative and accepted by the Conference. "That in future the Conference do not of necessity meet in the same town or at the same time as the British Association." Part of this resolution was put into effect in 1894 when the Conference was held at the end of July at Oxford, the town visited by the British Association later that summer. The other part was realised in 1895 when the Conference visited Bournemouth, the problem solving itself as no invitation was received from Ipswich, the British Association venue that year. From then on the Conference usually met at the end of July or early in August and any connections with the British Association were severed.

In 1900, London was visited for the second time following a pressing invitation given by the President of the Western Chemists Association of London, J. F. Harrington, and an offer from the Society to hold meetings in their premises. By this time the Conference usually extended to four days, including social functions.

In 1946, the Executive again considered the time of year when the Conference should be held. Late July was unpopular with seaside resorts and from 1947 onwards it has reverted to the original time of year, late August or as early in September as the host town can manage. The problem was discussed once more in 1958 but there is now no practicable alternative, particularly as the numbers attending have increased in recent years.

In Appendix D are set out alphabetically the 39 towns or cities in which the Conference has been entertained together with the years in which the Conference took place. The figures in brackets are the numbers said to be present, taken for the first few years from either the Proceedings or Minutes of the Executive, or later, by totalling those listed as present in the Year-Book. This latter practice ceased when the *Quarterly Journal of Pharmacy* replaced the Year-Book and figures are not shown again until 1948. Since that time the number shown is that reported to the Executive as the number of full-tickets sold. The totals do not, of course, reflect the numbers attending the science sessions!

Conference Sessions

Science Sessions

At the fiftieth meeting of the Conference it was reported that "with regard to the scientific work over 1,100 papers and notes have been contributed to its Scientific Sessions. These may be roughly grouped into (*a*) those dealing with an extension of our knowledge of the chemistry of drugs and (*b*) those designed to improve pharmaceutical preparations. In both cases this knowledge has had direct beneficial bearing upon the quality of drugs and hence upon the prestige of the craft". By 1962 the total number of papers presented at the Science Sessions was 2,168, including about 50 of a non-scientific character given before 1911 on

subjects which would have been discussed in later years at the separate Practice or Professional Sessions. Excepting for the War years the smallest number presented at any Conference was 14 and the largest 36, the latter in 1959.

The Executive has always had the right to reject papers submitted but the reasons for rejection appear to have varied from time to time. For example, for some 20 years from about 1874, papers of a non-scientific nature were held to be unsuitable. Authors of papers which were considered too "political", such as one on pharmaceutical remuneration in 1881, were persuaded to withdraw them. At times the Executive was careful not to discuss matters considered to be the province of the Society, but at other times, for example, during the period immediately before the First World War and as is mentioned below, under Practice Sessions, "political" papers were indeed encouraged. A number were rejected because they were medical papers dealing with subjects outside the pharmaceutical field; on at least two occasions the subject matter was regarded as unsuitable because it had been patented.

It has been the general policy to accept only papers describing original research work for discussion at the Science Sessions but there was little control of the quality of the papers until recent years when it has been accepted that the science papers should be of a standard suitable for publication in the *Journal of Pharmacy and Pharmacology*. This has, not surprisingly, led at times to differences of opinion with authors as the "standard" of a research paper is admittedly difficult to define. It must be a matter of opinion and as all papers are considered by several individuals the system has functioned with less friction than might be expected. Differences of opinion are likely to arise more often regarding the style and presentation than over the quality of the subject matter.

During the period 1870–1888 no less than 15 papers were read on aconite and aconitine. Other vegetable drugs and their active constituents which have been the subject of over 160 papers reported to the Conference over the years have been opium, cinchona, ergot, belladonna and other solanaceous drugs, and digitalis.

In 1866, a paper was read "On the results of the micro-chemical examination of extract of flesh" and a comment on the exhibition held that year read—"the exhibition proves one thing more, that there is a fashion in pharmacy as there is in dress. The present mode is undeniably Extractum Carnis". At that time "extractum carnis Liebig" and other concentrated extracts of beef were popular.

A note on siphon medicine glasses in 1868 contained the amusing sentence—"It has been thought that adults should not be entirely excluded the benefits of anything which facilitates or felicitates the deglutition of drugs, for it is astonishing how many a man will behave himself like a baby when he has a dose of Gregory under his nose".

The introduction of new synthetic organic substances into medicine during the latter half of the last century is, of course, reflected in the subjects of papers. For example, in 1869 there appeared the first of several papers on chloral. The title of another paper was "Carbolic acid

and human parasites" and a specimen of carbolic acid was shown—"of great beauty, exhibiting needle-like crystals of several inches in length". In 1875 "Some possible applications of salicylic acid in pharmacy" appeared, followed next year by three papers on salicylic acid and salicylates. In 1881, notes were read on heavy paraffin oil and shale and petroleum products. "Salol—a new antiseptic" appeared in 1886 and in the following year the pharmacist at the German Hospital discussed some synthetic compounds recently introduced including antifebrin, amylene hydrate and urethane. "The pharmacy of the thyroid gland" was read in 1893, a paper entitled "The composition of diphtheria antitoxin serum" in 1896 and a "Note on vitamines" in 1919.

The diversity of subjects discussed is illustrated by "Composition of the air of sewers and drains" in 1873, the determination of the fat content of milk in 1875 when a speaker said—"he would have rather not taken part in a tournament of public analysts, with whom it was rather early days to enact the tragedy of the Kilkenny cats", "Presence of metallic compounds in alimentary substances"—(copper in canned peas!) and the "Analysis of preserved carrots, potatoes, cabbage and mixed vegetables" in 1877—work carried out for the Admiralty following an outbreak of scurvy in an Arctic expedition.

"Tablet making at the dispensing counter" in 1896 was the first time a reference appeared to tablets in the title of a science paper. In 1910 papers were presented dealing with the bacteriological testing of disinfectants and Rideal took part in the discussion of these. A paper entitled "The composition of diabetic foods" presented in 1911, indicating that the claims made for a number of them were quite unjustified, was referred to the British Medical Association.

The first report to the Conference of drugs being tested on animals appears to be in a paper "New derivatives of codeia" in 1871, the products having been tried on cats. In 1874 the President, T. B. Groves, read a paper on an extractive from aconite and in reply to a question as to the evidence upon which he said it was inactive in animals he said—"The evidence is that I have taken it myself in ½ grain doses and that it has had no effect whatever; it is simply bitter!" In 1875 work on the physiological effect of an alkaloid from jaborandi was reported and the word "pharmacology" first appeared in the title of a paper in 1887—"The chemistry and pharmacology of some of the morphine derivatives". In 1922, during the discussion of a paper on strophanthus, a speaker suggested that the Society should establish a laboratory for the physiological standardisation of drugs—the Society's laboratories were established in 1926. As would be expected, just before and immediately after the publication of a British Pharmacopoeia, the subjects of papers and indeed the number of them were stimulated by pharmacopoeial problems.

From the beginning of this century greater interest was taken in the standardisation of the activity of drugs and preparations rather than in the mere improvement of the appearance of products or consideration of the adulteration of drugs. Reading the titles of papers presented between the Wars indicates that standardisation and developments in the analytical

field were the predominant influences affecting the subjects investigated by Conference research workers.

Presentation of Papers

Although the suggestion had been made on one or two earlier occasions, it was not until 1903 that by courtesy of the Editor of *The Pharmaceutical Journal* proof-copies of the papers to be read were made available to members at the annual meeting. When the Year-Book was incorporated in the *Quarterly Journal of Pharmacy and Pharmacology* the Editor of the latter provided the galley-proofs and ever since 1933 they have been available to members a few days before the Conference opens. This procedure, which has been possible through the generosity of the Society, has greatly facilitated the presentation and discussion of the papers.

Although it is recorded that in 1900 a paper was illustrated with lantern slides it was not until 1959 that projection facilities were made available routinely to authors to assist in presenting their papers. This change partly resulted from authors being permitted in 1958 to present "Short Communications" in addition to the normal complete papers. "Short Communications" were introduced following the practice of some other scientific bodies, to provide research workers with a forum for the discussion of research they were conducting which had not yet been completed and of problems they had encountered which would be of interest to others working in the same or similar fields. "Short Communications" are still in the experimental stage so far as this Conference is concerned but their introduction was an indication of the wish of the Executive to allow the procedures of the Conference to evolve so that they meet the changing needs of the scientific worker.

Sources of Science Papers

Most science papers have their source in university or college departments, or in industrial laboratories. The figures below illustrate this—the papers not accounted for were presented in the main by hospital pharmacists and public analysts.

Period	*Total No. of Papers*	*From Educational Institutions*	*From Pharmaceutical Industry*
1921–1939	440	182 (41 per cent)	216 (49 per cent)
1947–1954	190	71 (37 per cent)	111 (58 per cent)
1955–1962	232	130 (56 per cent)	93 (40 per cent)

It is gratifying that the increased facilities now available in educational institutions for post-graduate research are reflected in the larger number of papers presented to the Conference in the last few years from this source.

Practice Sessions

Practice Sessions were inaugurated in 1911 to provide formal facilities for the discussion of subjects related to the practice of pharmacy of a more general nature than those usually considered at the Science Sessions.

However, before 1911 a number of papers of a non-scientific type, the majority of which would have been considered suitable for Practice Sessions, were presented at Science Sessions. The subjects were diverse, including pharmaceutical ethics, relations with the Customs and Excise, accidental poisoning, pharmaceutical responsibility and remuneration, education, historical notes and a feature of several meetings and not entirely unscientific, a description of—"the salient features of the flora"—of the area in which the Conference was held, by the late Dr. Druce. Some of these papers are of interest.

In 1865, the subject of "Pharmaceutical Ethics" was included in the Research List. It seems probable that this was done to permit the presentation of a long paper with this title in the following year by Ince. He dealt with the subject in a comprehensive manner and the paper makes interesting reading today. He declared the shop should be called "a pharmacy", the customer should always be supplied with the exact article required and there should be no substitution. In referring to the "ethics of the shop" he wrote: "never forgetting the essentially trade nature which belongs to pharmacy, we at once come to the first ethical rule of the pharmaceutist, namely the necessity for the absolutely genuine character of his drugs". . . . "The principle which ought to guide the pharmaceutist in the regulation of his charges is that remuneration should increase in proportion as the class of article makes greater demand on the knowledge obtained by his professional education. If he sells articles dealt in by other classes of tradesmen, he must submit to the same rate of profit. In drugs proper, which require an educated judgment, power of testing and the like, he is entitled to a much higher rate; whilst in all matters of dispensing, his charges should be professional in their character, and not calculated on the cost of employed materials at all."

In 1869 reference was made in a paper on "pharmaceutical education in the provinces" to the Chair of Pharmacy in the faculty of medicine at Durham University, the Professor instructing both pharmaceutical and medical students—"the claims of pharmacy as a profession are for the first time recognised by an English University".

At the 1873 Conference a paper was read entitled "A proposal for a sign to be used by doctors ordering unusual doses on prescription". After discussion the following rules were agreed. The initials of the prescriber should be put against unusual doses; the name and address of the prescriber should be on the prescription; and it is desirable for such prescriptions to be retained by the dispenser.

Five hundred copies of these recommendations were printed and circulated widely.

In 1909, a discussion was arranged on "Should the dispensing of medical prescriptions be exclusively confined to pharmacists?" A resolution was passed asking the Executive to consider the subject and if thought desirable consult with the British Medical Association. This the Executive did and a conference between representatives of the two organisations was held to discuss medical dispensing, the nature and extent of prescribing by unqualified persons and the "possibility of co-operation in dealing with

problems bearing upon the sale of dangerous, secret or useless nostrums, advertised or otherwise, to the public". As a result a Joint Standing Committee was set up to promote the realisation of aims found to be common to both bodies.

In the following year this Committee discussed the mutual interests of doctors and pharmacists under the National Insurance Bill and suggested that dispensing under the proposed scheme should be by pharmacists, remuneration should be by a tariff system, there should be free choice of pharmacist by the patient and pharmacists should be represented on Local Health Committees. Unfortunately the B.M.A. Council did not accept these proposals but passed five not identical resolutions including "that dispensing should be by pharmacists or by medical men".

In 1911 when Practice Sessions were introduced the Executive decided that the Chairman of the Session should be a person appropriate for the subject under discussion and not necessarily the President. The phrase "practice of pharmacy" was minuted as including "all ethical, professional and educational topics but excluding commercial discussions". Between 1911 and 1921 seven series of Practice Sessions were held and 22 subjects were included in the programmes.

There were some eventful discussions at the Practice Sessions. In 1911 one subject considered was "Secret and Proprietary Medicines" following which the Conference resolved that a public inquiry into "the advertising and sale of proprietary secret remedies and the law relating thereto, with a view to further legislation for the prevention of fraud and quackery" was desirable. However, the resolution was not sent to the Government as it was learnt that the latter had already decided to hold an inquiry into the problem.

In the same year, following a discussion of the National Insurance Bill, telegrams were despatched to Members of Parliament and the Chancellor of the Exchequer asking for support for amendments to the Bill proposed by Mr. W. S. Glyn Jones, M.P.

It is of interest that a paper related to the one presented in 1873 mentioned above, entitled "Uniformity in the dispensing of abnormal prescriptions—a suggested code of rules" was discussed in 1914. It was hoped to obtain agreement with the British Medical Association on rules for both prescriber and dispenser but the matter was not actively pursued because of the War.

"Practice Sessions" were discontinued after 1921 and the Society arranged meetings of its Branch Representatives to discuss similar topics.

Professional Sessions

These have been held as part of the Conference since 1956 and the events leading up to their introduction have already been described. They have followed very closely the pattern of the Practice Sessions except that not more than one subject has been discussed at each session.

Symposium Sessions

In 1946 it was decided to include another type of science session—"to provide an opportunity for the review and discussion of some current

scientific subject of pharmaceutical importance". In these symposium sessions one or more experts introduce the subject—supplemented usually by prior circulation of their review—and it is open to all members to contribute to the discussion from their own experience. Sessions have been arranged at every Conference since and have proved very popular. The Department of Pharmaceutical Sciences of the Society is also arranging regular scientific meetings and discussions and there is now need for closer liaison between its Director and the Conference to avoid the possibility of the same or related subjects being chosen for discussion at or about the same time, so causing confusion.

Conference Lecture

In 1961 a further step in broadening the presentation of scientific matters to the Conference was the introduction of a Conference Lecture. It is of interest that the only previous occasion when a lecture was delivered to the Conference was in 1919 when F. H. Carr gave the first Harrison Memorial Lecture.

Social and Other Functions

One of the objects of the founders of the Conference was the promotion of "friendly reunion" among those engaged in the practice, or interested in the advancement of pharmacy. No difficulties arose in achieving this objective—the social events arranged during the annual meetings have always been a popular feature of the Conference. Indeed one of the concerns of the Executive over the years has been to control the enthusiasm of Local Committees in regard to both the cost of, and the time allocated to, the entertainment of members.

In 1872 the Executive minuted that while the Conference aims were promoted by gatherings of a semi-social, semi-scientific character, it was "not encouraged to any important extent by expensive entertainments". In the annual report for 1879 the unusual step was taken of congratulating the Sheffield Local Committee for taking cognisance of the views of the Executive and not arranging a formal banquet that year.

In 1884 it was decided that the members attending the Conference should pay for the luncheons, etc., supplied during the business days of the meeting and all travelling expenses involved if they took part in excursions. The annual report stated—"Your Committee have noticed with concern that the entertainment of visitors had a tendency to become more lavish year by year and that the cost might become a serious burden not only to local pharmacists, but to those residing a considerable distance from the place of meeting". Again in 1885 the Committee viewed—"with regret that at the present meeting a large sum will be thus expended".

No further references to the cost of the Conference appeared until 1924 when the Executive resolved—"that the programme of social events should not be unduly long and that the expenditure should be limited to the price of the tickets as nearly as local circumstances permit. Further . . . that Sports Day should be a separate item not included in the price of the

ticket". However, in 1936 the attitude to Sports Day changed and the sports competitions became part of the official programme. In recent years the view of the Executive in regard to the Conference Excursion has been that it should be restricted to a half-day event unless local circumstances are such that a full-day must be allocated to it.

In the early years exhibitions of "objects of interest relating to pharmacy" were held on a few occasions at the annual meetings, but even by 1870 it was remarked that the exhibits were "becoming rather commercial". Conversaziones were more common in the 1880's and 1890's at which scientific objects and photographs were shown.

The pressure of commercial interests to advertise their products during the Conference has been very wisely resisted by the Executive. In 1934 it was resolved—"That it disapproves of invitations by pharmaceutical firms, in the town in which the Conference is meeting, to visit works during the hours of Conferenec Sessions, or offers to distribute gifts to members attending the Conference".

The question of holding an exhibition during the annual meeting was reconsidered in 1952 and again in 1959 and on both occasions the Executive decided that the only form acceptable would be demonstrations organised at an educational institution in the town visited, of a non-commercial character, and arranged by the Local Committee or the educational institution, or both.

Summary

The founders of the Conference, at the inaugural meeting in 1863, could not have anticipated even in their wildest dreams, how soundly they were planning or how successful their organisation would prove to be. Their prime objectives were to hold an annual Conference, and to promote the advancement of pharmacy and the encouragement of pharmaceutical science. In 1963 this Conference is holding its one-hundredth annual meeting; despite two World Wars, a meeting has been held every year since 1863.

The methods adopted to encourage pharmaceutical research have been described; in addition to holding the annual meeting for the reading and discussion of papers they were, the making of grants to research workers, the publication of the Research List, the publication of the Unofficial Formulary, and the publication of the *Year-Book of Pharmacy*, including the Proceedings of the Conference.

One of the most valuable decisions taken in 1863 was to allow membership of the Conference to not only those engaged in the practice of pharmacy, but also to those interested in its advancement and not necessarily trained as pharmacists. Many of its leading personalities and staunchest supporters over the years have proved to be non-pharmacists.

The Conference provides a meeting place for all in pharmacy whether they be engaged in hospitals, teaching, industry, general practice or research work in disciplines allied to it. Further, the holding of these annual meetings enables pharmacy to be presented to the public in its

true light. It provides, in the modern idiom, the public image so sought after by public relations officers!

Let it not also be forgotten that the Conference has prospered through the voluntary effort of those who felt they had a duty to give something to their calling. This not only applies to those who have held office or contributed to the meetings but also to the thousands of members of Local Committees who have worked without seeking reward, except the gratitude of those attending the meetings they have helped to make so successful.

Provided the members are sufficiently far-sighted to allow the Conference to evolve and keep pace with changes in the methods of presenting and discussing scientific advances in the future, there would seem no good reason why it should not be just as successful over the next century as it has been in the past. The essential requirements are those stated in 1933 by the Chairman, C. H. Hampshire.

"The future of the Conference and its continuation as a useful body in the national life will rest principally upon its reputation as a means for the publication of scientific research in pharmacy, and for the discussion of technical problems."

References

Pharm. J., 1852, 1st series, **12**, 123–125.
Ibid., 1863a, 2nd series, **4**, 506.
Ibid., 1863b, 2nd series, **4**, 562.
Ibid., 1863c, 2nd series, **4**, 563–564.
Ibid., 1863d, 2nd series, **5**, 58–59.
Ibid., 1863e, 2nd series, **5**, 145–152.
Ibid., 1868, 2nd series, **10**, 131–132.
Ibid., 1955, 4th series, **175**, 455.
Yearb. Pharm., 1870, 348–349.
Ibid., 1872, 464.
Ibid., 1922, 443–455.

APPENDIX A

CONSTITUTION

Art. I. This Association shall be called the British Pharmaceutical Conference and its objects shall be the following.

1. To hold an annual Conference of those engaged in the practice, or interested in the advancement of Pharmacy, with the view of promoting their friendly reunion, and increasing their facilities for the cultivation of Pharmaceutical Science.

2. To determine what questions in Pharmaceutical Science require investigation, and when practicable to allot to individuals or committees to report thereon.

3. To maintain uncompromisingly the principle of purity in medicine.

4. To form a bond of union amongst the various associations established for the advancement of Pharmacy, by receiving from them delegates to the annual Conference.

Art. II. Membership in the Conference shall not be considered as conferring any guarantee of professional competency.

RULES

1. Any person desiring to become a member of the Conference shall be nominated in writing by two members, and balloted for at a general meeting of the members, two-thirds of the votes given being needful for his election. If the application be made during the recess, the Executive Committee may elect the member by a unanimous vote. Every member shall sign an obligation to conform to the rules of the Conference for the time being.

2. The subscription shall be 5s. annually, which shall be due in advance, upon July 1.

3. Any members whose subscription shall be more than two years in arrear, after written application, shall be liable to be removed from the list by the Executive Committee. Members may be expelled for improper conduct by a majority of three-fourths of those voting at a general meeting, provided that 14 days' notice of such intention of expulsion has been sent by the Secretaries to each member of the Conference.

4. Every association established for the advancement of Pharmacy shall, during its recognition by the Conference, be entitled to send delegates to the annual meeting.

5. The officers of the Conference shall be a President, four Vice-Presidents, a Treasurer, two General Secretaries, one Local Secretary, and nine other members, who shall collectively constitute the Executive Committee. Three members of the Executive Committee to retire annually by ballot, the remainder being eligible for re-election. They shall be elected at each annual meeting by ballot of those present.

6. At each Conference it shall be determined at what place and time to hold that of the next year.

7. Two members shall be elected by the Conference to audit the Treasurer's accounts, such audited accounts to be presented annually.

8. The Executive Committee shall present a report of proceedings annually.

9. The Rules shall not be altered except at an annual meeting of the members.

10. Reports on subjects entrusted to individuals or committees for investigation shall be presented to a future meeting of the Conference, whose property they shall become. All reports shall be presented to the Executive Committee at least *fourteen days* before the annual meeting.

The form of obligation mentioned in Rule 1 read as follows:—

"OBLIGATION

Having been elected a member of the British Pharmaceutical Conference, I hereby undertake to comply with all its rules."

APPENDIX B

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, being those holding similar rank in the Pharmaceutical Society of Great Britain and (*b*) corresponding members of the Conference.

3. Corresponding members of the Conference shall be persons at home and abroad interested in the advancement of Pharmacy who, not being qualified for membership of the Pharmaceutical Society, desire to be associated with the work of the Conference. A corresponding member shall be nominated in writing by a member, honorary member, or corresponding member, and elected by the Executive Committee.

4. The Officers of the Conference, who shall collectively constitute the Executive Committee, shall be:

A Chairman, Vice-Presidents, Vice-Chairmen, one Honorary Treasurer, two Honorary General Secretaries, together with nine others, being members, honorary members, or corresponding members of the Conference, at least three of whom shall be members of the Council of the Pharmaceutical Society. The President of the Pharmaceutical Society shall be *ex officio* a member of the Executive Committee and the President of the Conference. The Honorary Local Secretary shall be *ex officio* a member of the Executive Committee.

5. 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 28 days before the annual meeting.

6. 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.

7. Members, honorary members and student-associates shall not be required to pay a separate subscription to the British Pharmaceutical Conference; they shall be entitled, on application, to a copy of the *Year-Book*. Corresponding members of the Conference shall pay a subscription of 10s. 6d. annually, which shall entitle them to a copy of the *Year-Book* on application; such subscription shall become due on January 1.

APPENDIX C

REVISED CONSTITUTION AND RULES

1. The British Pharmaceutical Conference is an organisation associated with the Pharmaceutical Society of Great Britain, which exists for the purpose of discussing subjects relative to the science of pharmacy, promoting friendly reunion among pharmacists and those interested in pharmacy and generally furthering the objects sought to be obtained under the Royal Charters granted to the Pharmaceutical Society of Great Britain. The Conference shall meet annually.

2. The Conference shall consist of:

(*a*) Fellows, members, honorary fellows, honorary members and registered students of the Pharmaceutical Society of Great Britain;

(*b*) members of the Pharmaceutical Society of Ireland and members of the Pharmaceutical Society of Northern Ireland while these Societies remain associated with the Conference and pay annual contributions to the Conference in lieu of individual subscriptions from their members;

(*c*) elected members.

3. Elected members are those persons at home and abroad interested in subjects relative to the science of pharmacy who, not being members of the Pharmaceutical Society of Great Britain or one of the other Societies associated with the Conference, have been elected by the Executive.

Proposals for membership by election must be made in the form approved by the Executive Committee and must be signed by three members of the Conference who recommend the candidate as a fit and proper person to be associated with the Conference.

4. The Officers of the Conference shall consist of a President, a Chairman, an Honorary Treasurer and two Honorary General Secretaries.

The President of the Pharmaceutical Society of Great Britain shall be the President of the Conference; the other officers of the Conference shall be nominated by the Executive Committee, and the nominations shall be subject to the approval of the annual meeting of the Conference.

5. The Executive Committee of the Conference shall consist of the Officers of the Conference, five past-Chairmen who have most recently filled the office of Chairman, together with three persons nominated by the Council of the Pharmaceutical Society of Great Britain and nine other members 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 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. Student members may not be members of the Executive Committee.

6. Of the nine other members of the Executive Committee for whom provision is made in Rule 5, three shall retire each year in accordance with seniority of service or otherwise as may be decided at the annual meeting of the Conference. Such retiring members shall be ineligible to serve again until after the lapse of one year. The vacancies so created shall be filled by election at the annual meeting of the Conference. Nominations shall be made by the Executive Committee, due regard being paid to the desirability of securing representation of the various branches of pharmaceutical practice and its associated scientific disciplines. Nominations may also be made by any five members of the Conference, in writing, to the Honorary General Secretaries at least 28 days before the commencement of the annual meeting. In the event of there being more nominees than vacancies a ballot shall be held in accordance with arrangements to be made by the Executive Committee. Any casual vacancy shall be filled by co-option.

7. The Chairman of the Conference shall *inter alia* give the inaugural address, preside over the scientific meetings and take the chair at the meetings of the Executive Committee.

8. Non-members of the Conference may attend the annual meeting of the Conference only as guests of members or at the invitation of the Executive Committee. They may not vote at any business session of the Conference. The Executive Committee may, however, decide that attendance at any particular session or function is to be restricted to members of the Conference. Student members of the Conference may not vote at any business session of the Conference.

9. Elected members shall pay an annual fee which will be decided from time to time by the Executive Committee. Subscriptions shall become due on January 1, and membership shall cease if subscriptions are not paid by June 1.

10. By arrangement with the Pharmaceutical Society of Great Britain, the transactions of the Conference are published in the *Journal of Pharmacy and Pharmacology*. Members of the Conference may obtain copies of the Journal on preferential terms.

APPENDIX D

PLACES OF MEETINGS

Aberdeen	1885 (151)	1908 (212)	1932	1955 (643)	
Bath	1864 (60)	1888 (155)	1924 (350)		
Belfast	1898 (214)	1935			
Birmingham	1865 (106)	1886 (188)	1906 (275)	1939	
Blackpool	1949 (461)				
Bournemouth	1895 (130)	1936	1959 (754)		
Bradford	1873 (104)				
Brighton	1872 (137)	1905 (234)	1927 (420)	1948 (387)	
Bristol	1875 (118)	1903 (187)	1957 (659)		
Cambridge	1910 (207)				
Cardiff	1891 (118)	1930			
Cheltenham	1928				
Chester	1914 (274)				
Dublin	1878 (147)	1901 (229)	1929	1956 (882)	
Dundee	1867 (67)	1902 (199)			
Edinburgh	1871 (116)	1892 (211)	1912 (413)	1938	
Exeter	1869 (69)				
Glasgow	1876 (176)	1897 (293)	1925 (450)	1950 (568)	
Harrogate	1951				
Hastings	1884 (80)				
Leeds	1890 (154)	1934			
Leicester	1926 (310)				
Liverpool	1870 (170)	1896 (184)	1920 (370)	1937	1962 (650)
Llandudno	1958 (630)				
London	1874 (175)	1900 (234)	1913 (525)	1915	1916
	1917	1918	1919 (121)	1923 (514)	1933
	1940	1941	1942	1943	1944
	1945	1946	1953 (691)		
Manchester	1887 (238)	1907 (370)	1931		
Newcastle upon Tyne	1863 (21)	1889 (170)	1909 (211)	1960 (611)	
Norwich	1868				
Nottingham	1866 (84)	1893 (172)	1922 (257)	1952	
Oxford	1894 (163)	1954 (461)			
Plymouth	1877 (137)	1899 (146)			
Portsmouth	1911 (101)	1961 (712)			
Scarborough	1921 (282)				
Sheffield	1879 (148)	1904 (199)			
Southampton	1882 (119)				
Southport	1883 (186)				
Swansea	1880 (77)				
Torquay	1947				
York	1881 (168)				

SYMPOSIUM

FINE PARTICLES IN PHARMACEUTICAL PRACTICE

CLINICAL AND PHARMACEUTICAL ASPECTS

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Introduction

While the fundamental factors required to explain the effects of particle size were first investigated in the last century, the technology of particle size is much more advanced in some industries, for example, the manufacture of paints, cement and ink, than in others; which, as yet, must include pharmacy. Scant attention has hitherto been paid to particle size effects, whether in official reference books or in text books of pharmacy, and a relatively insignificant research effort has been applied to this and related topics in pharmacy. The effects on therapeutic efficiency and on presentation, stability and control procedures has now been recognised.

The commonly accepted criterion for a dose, namely an amount by weight of a drug, is not the only factor necessary to ensure a consistent pharmacological response; the size of the particles of some drugs has a considerable bearing on their efficacy. In one instance at least, that of griseofulvin, control of fineness has led to a dosage scheme half of that formerly required.

The significance of the fineness of small particles in pharmacy can be conveniently considered from clinical, pharmaceutical, manufacturing and control aspects. In this review, those aspects of pharmacy in which the effects of solid particle size is important will be discussed.

Clinical Aspects

The infinitely complex biochemical system that we are seeking to influence by administering drugs comprises factors too numerous to allow their inter-relationship to be followed adequately. At this early stage in the understanding of the effects of particle size, we can only feel our way empirically towards the more effective presentation of drugs.

(Reviews and theoretical treatments of drug absorption and distribution have been made by Teorell, 1937, and Wagner, 1961).

Oral Therapy

Drugs absorbed in solution for systemic effect. Drugs are normally absorbed in solution from the gut. The absorption process can be rapid enough to make the solution rate of the drug the rate-determining step in the therapeutic process; limitation of absorption at this point is confined to drugs of low solubility.

There are many examples of drugs whose particle size influences their uptake from the gut. Corticosteroids and their relatively insoluble esters taken orally in tablets have been reported to give variable clinical results;

although a varying rate of disintegration was thought at the time to be the explanation, it is now accepted that the difference among preparations was due to differences in particle size.

Sulphur is practically insoluble in water; most of the dose of powdered sulphur given orally passes through the bowel unchanged (Wild, 1911), and only 10 per cent of the dose given to dogs was absorbed (Denis and Reed, 1927). Orally administered colloidal sulphur is rapidly and completely absorbed, to give peak urinary levels of sulphur compounds within 2 hr. Powdered sulphur (100 mesh) is poorly absorbed, the peak occurring 8 to 16 hr. after ingestion, at which time it is probably undergoing chemical modification in contact with the intestinal flora of the lower bowel (Maillard, 1911, Greengard and Woolley, 1940). A macabre demonstration of the efficient absorption of colloidal sulphur was obtained when concentrates of a water from Graham Springs, Kentucky, containing colloidal sulphur taken orally in large doses, caused intoxication and death within 5 min., indicating that the absorption of sulphur in this form is exceedingly rapid.

Sulphur, therefore, is an example of a drug whose particle size has a profound effect on efficiency of absorption, and may also modify the processes by which absorption occurs and can even lead to toxic effects.

Sulphonamides, in general, have a limited solubility in water. The greater efficiency of micro-crystalline sulphadiazine, each crystal approximately 1/350th the mass of normal crystalline material, has been demonstrated by Rheinhold, Phillips and Flippin (1945), and the marked influence of the effects of food or fasting state of the subject on uptake of the micro-crystalline form but not, apparently, of the control crystalline material noted. The importance of checking the effects of particle size on absorption under various conditions of fasting and food intake, and the need to arrange for doses to be given in precise relationship to food intake, should be recognised when planning such experiments.

The efficiency of absorption of the antibiotic griseofulvin depends on particle size, and a direct relationship between the logarithm of the specific surface and absorbability has been established (Atkinson, Bedford, Child and Tomich, 1962). The work, greatly aided by the availability of a precise method for assaying the drug in blood samples, led to appreciable savings in treatment cost, this being one of the justifiable reasons for doing research in this field.

Conflicting pharmacological responses can be obtained unless due attention is paid to the fineness of drug particles.

The physiological effects of *p*-hydroxypropiophenone (PHP) in animals were in dispute for a time. Whether the substance had oestrogenic activity or alternatively an inhibitory action on the secretion of gonadotrophic hormone was the problem. Impurities in certain samples were thought to explain the conflicting results, but the contradiction was finally resolved only when careful experiments were conducted on rats with samples of PHP specially prepared in a range of crystal sizes (Foglia, Penhos and Montuori, 1955). Although an initial preparation of PHP did not provoke oestrus or inhibit the action of the pituitary in rats, even

when administered in high doses for a long time, the same powder reduced to crystals of 2,000μ^3 or less produced the typical effects of an oestrogen. These small crystals when reconverted to larger ones (3,000 to 10,000μ^3) became inactive.

Drugs absorbed as solid particles. Bacteria pass through the wall of the alimentary tract, and particulate material can do likewise. Resin particles, 1–5 μ in diameter passed through the mucous membrane of the tonsil, pharynx and small intestine of the calf (Payne, Sansom, Garner, Thomson and Miles, 1960). Little of the dose was absorbed, but the factors governing such absorption have not yet been studied in detail.

Drugs acting locally in lumen of gut. Some drugs, administered orally, are required to exert their action within the lumen of the gut, e.g., anthelmintics, amoebicides, contrast agents and some antibiotics and sulphonamides.

Attempts to improve the efficiency of anthelmintics by reducing the particle size have led to conflicting possibilities. Although the primary objectives of higher solution rate for the drug in the gut and improved dispersion of the solid particles can be achieved, the consequences of the concommitant increased rate of absorption (and hence removal) from the gut must be borne in mind (Swales and Collier, 1940). Improvement in clinical efficiency may not result from increased solution rate; indeed, particle size reduction can reduce the efficacy of these drugs. Toxicity from the absorbed drug can become important when the particle size of the drugs intended to act within the gut is reduced.

Improved *in vivo* anthelmintic action of small particle phenothiazine is well established (Kingsbury, 1958); particles with equivalent sphere diameters less than 10 μ are desired, whereas particles greater than 20 μ in diameter have little anthelmintic action. Similar results have been obtained with bephenium hydroxynaphthoate and bephenium embonate, for which it appears that 30 μ is the limiting diameter for good anthelmintic effect (Newman and Axon, 1961). Presumably these insoluble salts of bephenium were chosen to minimise the absorption from the gut that might occur with more soluble salts.

A drug evaluated for use orally in the veterinary field is 4,4′-dinitrocarbanilide, for which particles of 3 to 5 μ have been shown to be less effective than those under 1 μ in diameter (Merck).

Barium sulphate for radiography of the alimentary canal should be fine but it is possible for extremely small particles to give rise to unexpected results. Small particle barium sulphate administered as an enema can enter intestinal glands and may be the cause of barium granulomas (Sasson, 1960). Similarly, silica gel particles have been shown to produce intestinal nodules in rats, and it seems likely that particle size of the silica will determine the extent of this occurrence (Desai, Burkman and Salisbury, 1958).

Parenteral Therapy

The fineness of the particles of an injected drug can influence the rate of absorption from the injection site, and this has been recognised for

zinc insulin in the British Pharmacopoeia, although the physical nature of the insulin is also highly important. Amorphous zinc insulin produces a rapid effect, whereas crystalline zinc insulin in suspension produces a prolonged effect, particularly if care be taken to ensure that a substantial proportion of the crystals are longer than 5 μ (Hallas Møller, 1954).

Particle size of procaine penicillin is important in injectable suspensions, the extent of the effect depending on the nature of the vehicle used. Arachis oil, as a gel with aluminium stearate, prepared so as effectively to coat the procaine penicillin particles with a hydrophobic protective layer, gives prolonged blood levels with small particles of procaine penicillin, preferably below 5 μ (Buckwalter and Dickison, 1958). On the other hand presentation of procaine penicillin in extremely small particles in an aqueous suspension could have the opposite effect of increasing the rate of uptake of antibiotic from the site of injection.

The particle size of drugs in solution for injection containing high concentrations of solid may influence blood levels by modifying viscosity and thereby minimising spread of the injection in the muscle (Ober, Vincent, Simon and Frederick, 1958).

The relationship between crystal size and oestrogenic activity of *p*-hydroxypropiophenone mentioned earlier, applies equally to the same compound injected subcutaneously (Foglia, Penhos and Montuori, 1955).

Modification of the release of drugs from injected suspensions by control of particle size has been applied to drugs that cannot readily be prepared in crystalline form (Organon). To obtain particles of the required size, the drugs (adrenocorticotrophic, gonadotrophic and thyrotropic hormones, testosterone and heparin) were first compressed to glassy solids by the application of high pressures and subsequently reduced in size by milling and graded by sifting.

Rectal Therapy

Particle size can influence the absorption of drugs administered in rectal suppositories. Maximum rate of transfer from the oily suppository vehicle to the aqueous mucosal fluids can be achieved by choosing a vehicle in which the drug has a low solubility and also by using a dispersion of fine particles to ensure that saturation is maintained in the melted suppository (Reigelman and Crowell, 1958).

Topical Treatment

Many ointments contain dispersed particles of drug that exert their effect by solution in the base and transfer by partition to the skin or wound exudate.

The processes involved in securing therapeutic effect when such ointments are applied to the skin are: (i) solution of drug in base; (ii) diffusion of drug through base to skin; (iii) transfer of drug from base to skin; (iv) diffusion of drug through skin.

Maximum possible concentration of the drug in solution in the base at the ointment-skin interface is desirable, to encourage transfer of the

drug from the ointment to the skin. Maintenance of maximum concentration in the ointment at the interface depends on the relative rates of processes (i) and (ii) compared with processes (iii) and (iv). Presentation of the drug in fine particles in the ointment base minimises the possibility that solution and diffusion processes in the base will limit the clinical response. That this is so can be appreciated when it is remembered that corticosteroid ointments containing as little as 0·01 per cent w/w of dispersed drug are now being used.

Increased inhibitory effects have been demonstrated for ammoniated mercury and yellow oxide of mercury when present as small particles in standard ointment bases (MacDonald and Himelick, 1948). Similarly, the activity of calomel ointment has been shown to depend on the particle size of the dispersed drug.

The treatment of superficial burns with sulphathiazole in microcrystalline form gives higher rates of solution, improved dispersion and freedom from clumping or caking on the burn (Sharr, Ferguson and Nova, 1942). The same type of concentrated sulphathiazole magma of fine particles gave much improved results in impetigo contagiosa when compared with conventional dusting powders or ointments containing the same drug (Harris, 1943).

Some ointments, such as those containing calamine or zinc oxide, are required to provide protective and reflective properties. For these the reflective properties will depend on the particle size of the dispersed material, although for these preparations in particular the effect is not of great practical importance; thus calamine, being cheap, is not normally used at limiting concentrations.

Respiratory Tract Therapy

Treatment of the respiratory tract with aerosols containing solid particles is well established, especially for corticosteroids. The drug may be required to act in the trachea, bronchioles or alveoli. The mass of an individual particle largely determines how far down the orobronchial tree it will travel. Careful formulation is therefore needed if the steroid is to penetrate to the desired site and exert maximum and consistent effect. For clay dusts, maximum retention in the upper respiratory tract occurs when particles are 5 μ or more in diameter but particles less than 1 μ in diameter are not retained. On the other hand, satisfactory retention in the alveoli was achieved with much smaller particles, thus providing a basis for selective distribution of drugs according to particle size.

For xylocaine used in a pressurised aerosol, particles of about 5 μ diameter best ensure deposition on the mucosa throughout most of the tracheobronchial tree. Deposition on the pharyngeal mucosa occurs with larger particles, or in the alveoli if smaller particles are used (Tomashefski, Nelson and Christoforidis, 1962).

PHARMACEUTICAL ASPECTS

The pharmacist is the person best fitted to co-ordinate and interpret all the observations about particle size of drugs that can be collected.

There are, however, certain features, for example, particle size, of particular types of products that he alone will have to consider, assessing their significance and, if necessary, taking steps to control them.

Powders

The need to control the particle size of substances of limited solubility in aerosols, insufflations, dusting powders, and so on, can be forecast from results obtained with insecticides, which offer many instances of its importance.

Thus the toxicity of Paris Green particles to the Mexican leaf beetle has been shown to depend on particle size; Paris Green with an average particle diameter of 1·1 μ caused higher mortalities than did particles of average diameter 12 μ which were more effective than particles of diameter 22 μ. It was apparent that, for clear-cut results, it was important to ensure that each sample contained a narrow distribution of size of particles (McGovran, Cassil and Mayer, 1940).

Some substances are presented as powders to be made into extemporaneous solutions or suspensions by the clinician or pharmacist. Clearly there is a need for such products to behave uniformly and disperse readily in the shortest possible time. The character of the film of saturated solution at the solid-liquid interface largely determines dispersion and solution characteristics. For solids whose saturated solutions are of a syrupy consistency, too great a reduction in particle size is to be avoided, because the interfacial film of dissolving drug can cause the particles to adhere together, which will greatly reduce solution rate. Insoluble components, such as procaine penicillin, suitably dispersed as fine particles along with the more water-soluble components, such as streptomycin sulphate, minimise these difficulties.

The flow properties of powders depend on particle size, shape and density (Train, 1960). Reduction in particle size results in poorer flow properties, which means that blending and mechanical subdivision of fine powders is usually difficult. Griseofulvin and procaine penicillin are particularly troublesome to feed to mills or to fill into vials in small amounts mechanically at high speed.

Extremely fine powders with high specific surface areas can adsorb relatively large quantities of water. Synthetic silicates can adsorb three to four times their weight of water and yet behave as dry free flowing powders (Johns Mansville Celite Division, 1960).

Tablets

The fineness of a drug affects the likelihood of obtaining a satisfactory distribution of drug in each dose of powder or tablet preparation. Train (1960) has calculated that if a blend of two powders in equal amounts is required to be made such that there are 997 chances out of every 1,000 that either component will be within ±10 per cent of the true concentration, that is, actually between 45 to 55 per cent concentration, 800 particles must be present in each dose. If the permissible limits are set at ±1 per

cent of the intended value of 50 per cent, then 80,000 particles per dose would be required, and for a drug present at a nominal concentration of 1 per cent at least eight million particles per dose would be needed to maintain the desired limits of ± 1 per cent of the stated content.

The apparent intensity in colour of a powder depends on its particle size; indeed for coloured drugs, presented as uncoated tablets, it may be necessary to place some control on the particle size to avoid too much variation in colour from batch to batch.

Pharmacists use colour to make products more attractive to patients. Dyes adsorbed on aluminium salts as lakes are gaining in popularity for colouring coated or uncoated tablets. The lake should be in the form of fine particles to achieve uniform dispersion of colour. Different batches of coloured powder mixtures will vary in colour if the particle size of the highly coloured components are not controlled. Samples of carbon black with specific surfaces ranging from one to tenfold showed a similar difference in tinting strength when tested by a standard method (Herdan, 1960).

Presentation of finely powdered substances in tablet form requires the tablets to disintegrate so as to provide the original powder for solution. The criterion for a satisfactory conventional tablet is that the blood levels of drug obtained should be the same as those obtained by administration of an aqueous suspension or, say, of cachets containing an equivalent dose as a powder.

Suspensions

The viscosity of suspensions with high solid: liquid ratios depends on the particle size of the dispersed solid. Smoluchowski (1916) claimed that viscosity depends on, among other things, particle diameter and increases with decreasing particle size: this effect was confirmed by Kruyt (1922), for sulphur sols by Oden (1931), for ultramarine particles of around 5 μ diameter by Pryce Jones (1947) and for suspensions of metal carbonates and sulphates (10 to 40 μ range in diameter) in water by Ward and Kammermeyer (1940).

The relative viscosity of suspensions increases as the size ratio of the dispersed powder decreases, at least for suspensions containing more than 25 per cent of solid by volume. Styrene-butadiene latices consisting of spherical polymer particles have been examined; the conditions required for minimum viscosity were that the distribution of latex particle size should be as wide as possible and that the mass of the larger particles should comprise about 75 per cent of the total mass of the particles. This conclusion is in agreement with theoretical calculations showing that a minimum void volume is obtained when approximately 70 per cent of the mass of the particles is contributed by the larger of two particles. The degree of anisotropy of the particles in suspension may mask the effect of the size ratio of the powder; this is particularly so for particles of highly irregular shapes (Ward and Whitmore, 1950). A pharmaceutical system in which anisotropy of the particles masks the effect of size ratio is Injection of Propyliodone, B.P., particularly the aqueous injection, which

contains 50 per cent w/v solid material prepared by milling needle crystals of propyliodone.

Adjustment of particle characteristics in a suspension can enable a minimum viscosity to be obtained, but such a system will not necessarily exhibit minimum thixotropy.

Particle Size and Solubility

The solubility of small solid particles in a liquid depends on their size. Small particles are in equilibrium with higher concentrations of solute than are larger particles.

The relationship between the solubility of amorphous silica and its particle size has been determined and shown to be expressed by

$$\log S = 4{\cdot}80 \times 10^{-4} A - 2{\cdot}043$$

where S = solubility per cent at 25°; and A = specific surface m.^{2}/g. (Alexander, 1957).

The higher solubility of small particles, and the fact that solutions are in equilibrium with one size of particle only, must be borne in mind during experimentation (Higuchi, Rowe and Hiestand, 1963).

Phenolphthalein is an example of a drug for which the colloidal form has been shown to have a greater solubility than the crystalline form and also to be more active in producing its specific effect of bowel evacuation (Fantus and Dyniewicz, 1935).

Noyes and Whitney (1897) conducted a simple and elegant experiment on the rate of solution of benzoic acid and lead chloride and concluded that the rate at which a solid substance dissolves in its own solution is proportional to the difference between the concentration of that solution and the concentration of the saturated solution. They regarded the solids as being surrounded by an infinitely thin film of saturated solution and the process of solution being the dispersion of this film of saturated solution throughout the bulk of solvent.

The greater the available surface the greater the proportion of saturated film, hence the greater the solubility rate. Wilhelm, Conklin and Sauer (1941) calculated an effective diffusion film thickness of 0·016 mm. for sodium chloride crystals in specified conditions of agitation.

The different concentrations of solute in equilibrium with small and large particles of suspended solid results in migration of solute by diffusion from the "atmosphere" surrounding small particles to those of larger particles, the net result being that larger particles tend to grow in size at the expense of the smaller. This, the Ostwald effect (Ostwald, 1900), can cause the specific surface of powders in liquid suspensions to change on storage.

For each solid there will be a maximum size, depending on the composition of the suspending fluid, above which the effect will not be detectable. For gypsum the effect disappeared for particles above 5 μ diameter (Jones and Partington, 1951). The tendency for sulphadiazine crystals to grow in aqueous suspension has been examined mathematically (Hasegawa and Nagai, 1958).

Recent advances in instrumentation have led to improved methods for examining crystal growth and methods sensitive to less than 0·1 μ have been applied to methyl prednisolone (Higuchi and Lau, 1962).

Stability of Fine Particle Drugs

Reduction in particle size can cause faster deterioration of a solid drug stored in the "dry" state. The toxic components of pyrethrum deteriorate most rapidly upon exposure to light and air when the insecticide is finely ground. A measure of protection against the effect of light was obtained by coating pyrethrum particles with tannic acid, titanium dioxide or an antioxidant (Smith, 1936).

The decomposition of aspirin in the solid state has been investigated. A mechanism for this deterioration has been postulated, involving an initial adsorption of a water layer on the particle surface and diffusion of aspirin, with subsequent hydrolysis in the surface solution. Particle size is therefore likely to affect the decomposition (Leeson and Mattocks, 1958).

MANUFACTURING ASPECTS

Chemists and physicists both need to contribute their specialised fundamental understanding of natural phenomena towards the more efficient working of production processes. We are better equipped to understand what is going on in the manufacturing plant than in the human body.

Milling

Pharmacists frequently mill powders; the hazards involved in such processes must be kept in mind and suitable precautions taken. Atmospheric dusts of fine powders may explode violently given the proper concentration of solid and a source of ignition which the powder itself may provide by virtue of static electrical charge. The rate of the explosive reaction depends on the particle size of the solid phase. The relative inflammability of potato starch increases slowly as the particle size is reduced from 100 μ to 40 μ in diameter, but below this size the hazard rises steeply. For oat and corn dust and wheat flour inflammability hazards appear to be maximal just below diameters of 40 μ (Boyle and Llewellyn, 1950).

The ability of a powder to sustain an explosive reaction can be measured in the laboratory, and a classification for a particular powder may be obtained by submitting samples to the Safety in Mines Research Establishment, H.M. Factory Inspectorate, Ministry of Labour and National Service, Harper Hill, Buxton, Derbyshire.

For many pharmaceutical purposes it is important that the bulk density of a powder preparation should not fluctuate too widely. Dr. Heywood, in his conjoint paper, refers to the relationship between the size and packing of particles and the effect on the bulk density of a powder. Pharmacists experienced in the devices of production sometimes utilise the principles of inter-particle packing by subjecting to a further size reduction a proportion of a batch of powder with an unacceptable low bulk density

finally reblending with the remainder of the batch to yield a mixture of increased bulk density.

The influence of the type of mill and the conditions of their operation on the particle size of powdered materials is outside the scope of this review.

Polymorphic Solids

The process of reducing solids to fine particles can modify crystalline structure, which in turn can influence both solubility and rate of solution. For example, methyl prednisolone in one physical form is 1·8 times as soluble as another physically more stable form (Higuchi, Lau, Higuchi and Shell, 1963). The several forms of oestradiol, barbiturates and aluminium trihydroxides are readily interconvertible by apparently innocuous grinding techniques (Smakula, Gori and Wotiz, 1957, Cleverly and Williams, 1959, and Yamaguchi and Sakamoto, 1959). The infra-red spectra of steroids, benzoic acid and carbohydrates have been shown to change according to the method adopted for preparing the sample for examination (Roberts, 1957, Farmer, 1955, and Barker, Bourne, Weigel and Whiffen, 1956).

Analytical Considerations

Much that is relevant to analytical control methods is contained in the conjoint paper by Dr. Heywood. The analyst has a vital role to play in helping to devise and choose methods of determining particle size so as to control adequately particular characteristics as required.

The specific surface (air permeability) of griseofulvin has been found to correlate with blood levels and this variable is convenient to use for control purposes. Methods of determining specific surface area by air permeability, although not a measure of total surface area, can usually be expected to be adequate for drugs designed for oral use. Indeed, other methods, for example those involving nitrogen absorption, may measure powder surface not readily available for dissolution in an aqueous system; thus fissures and minute faults in crystals are measured by nitrogen absorption, but not by air permeability.

The possibility should always be considered that two powdered samples of a drug with identical specific surface areas but with entirely different distributions of particle size may give different rates of absorption from the alimentary canal.

Table I sets out comparative dissolution rates for two "theoretical" powders with the same specific surfaces. The rate of solution expressed per unit area of particle surface is assumed to be identical for large and small particles. Dose A comprises one hundred particles of 10μ in radius, Dose B contains one oversize particle, $40\,\mu$ in radius, with a sufficient number of a calculated second size to satisfy the requirements that the specific surfaces of both samples should be virtually identical.

Thus, a sample of a drug with particles of different sizes will dissolve more slowly than the same dose of drug containing particles of one size only, each sample having the same initial specific surface area. In

practice this means that, for oral use, drugs of controlled specific surface should preferably have a minimum distribution of particle size.

The influence of distribution of particle size on efficiency of absorption from the gut will be maximal for drugs given in large doses and absorbed solely in the upper parts of the alimentary tract. This effect was found not to be significant for griseofulvin, which is absorbed throughout the gastrointestinal tract (Atkinson, Bedford, Child and Tomich, 1962), but this is not true of other drugs. Crystals of phenothiazine have been seen in the faeces of sheep (Gordon, 1956). If, for the drug used as an example in the table above, the physiological conditions were such that particles of 10 μ radius were the largest that could be dissolved before the drug passed the "zone of absorption" in the gut, dose B would be only 75 per cent as effective as dose A.

TABLE I

Dose	Number of particles (spheres)	Particle radius (microns)	Total relative surface area per dose	Total relative weight per dose	Relative weight remaining after dissolution of increments of radius (μ)						
					−1	−2	−3	−4	−6	−8	−10
A	100	10	10,000	100,000	72,900	51,200	34,300	21,600	6,400	800	0
B	1	40	1,600	64,000	59,330	54,880	50,650	46,660	39,310	32,750	27,000
	457	429	8,412	36,090	16,280	5,487	981	0	0	0	0
			10,012	100,090	75,610	60,367	51,631	46,660	39,310	32,750	27,000

Control of particle size may be required for other reasons, such as to avoid needle blockage with injectable suspensions or to ensure reproducibility of viscosity in a suspension. For the former requirement it is usually adequate to supplement the determination of specific surface by a simple test for oversize particles, for example, by sifting an appropriate weight of powder as a suspension in a non-solvent liquid through, say, a 325 or 250 mesh sieve and examining the residue, if any, under the microscope.

Control of fine particle characteristics for suspension products containing high solid-liquid ratios can become complicated if an attempt be made to measure mean size, size distribution and degree of anisotropy of the particles and then relate these to ultimate effect on the suspension. The relationship between the three variates is complex and not fully understood; it is probably different for every suspension. In these circumstances the quality of the fine particle powder may best be controlled by setting up an empirical test embodying the essential features of the product system (omitting stabilisers, preservatives and so on, but including wetting agents, if used) and measuring viscosity directly in the manner used for the final product.

Conclusion

Particle size is important in many branches of pharmacy, so much so for some drugs, the marginally soluble ones, that it will not be inapt to consider the subject as a new technology. It has as much claim to this as rheology, in America the term "micromeritics" has already appeared.

To understand the significance of the many effects of fine particles requires explanations in physico-chemical terms. Examination of the effects of fine particles in pharmaceutical systems can provide fruitful and rewarding subjects for research.

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THE EVALUATION OF POWDERS

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Materials in powder form exhibit so many unique properties that they could be regarded as constituting a fourth state of matter. Compacted by vibration and pressure, they exhibit the rigidity of a solid body, if rounded in shape and suitably graded they will flow like a liquid, whilst if of very small size the particles link into chains forming an open system of the same order of density as a gas. Powder particles are normally solid, though the more general term of particulate matter is not so restricted and may apply to systems in which the particulate matter exists in any phase, i.e., as liquid particles in an emulsion or in a mist, or even as a system of gas bubbles in a liquid.

Pharmacy, however, is mainly concerned with particles in the solid state and this paper is restricted to a consideration of such materials. The field of powder technology study owes its fascination to the fact that there are many fundamental particle properties which apply to powders used in all industries, and yet each industry has specific problems of its own.

It is not easy to define an upper limit to the size of an individual particle, for a particle is a discrete portion of matter which is small in relation to the space in which it is considered, and the absolute size of the particle is irrelevant. Particulate materials, or powders in the present context, consist of a number of particles which may be dispersed or may be in loose contact though still retaining their individuality, i.e., do not adhere to each other. Such systems may be static or in motion. For practical purposes, however, an arbitrary definition of the size of powder particles is necessary, and B.S. 2955 states that a powder shall consist of discrete particles of dry material with a maximum dimension less than 1,000 μ (1 mm.). This definition merely puts an upper limit to the size of powders and methods of determining the size distribution of the constituent particles must be developed for the purpose of devising industrial specifications or for research on their properties. Before such methods of analysis are described, however, the basic fundamental properties of particles will be defined and discussed, since it is upon these principles that size analysis methods are founded.

Fundamental and Derived Properties

The manifold properties of powders can be elucidated only by making a distinction between those fundamental properties which can be defined individually, and derived properties which depend on some combination of the fundamental properties together with other factors such as the chemical constitution and the circumstances of usage. These fundamental properties are:

(i) The size of an individual particle.

(ii) The size distribution and the mean size of a system of particles.

(iii) The particle shape.

(iv) The particle density, including the influence of porosity.

Relatively simple examples of derived properties are:

(i) The specific surface.

(ii) The bulk density.

(iii) The terminal velocity of fall in a fluid, as used for the purpose of sizing analyses.

There are, however, much more complex properties concerning the applications of powders, such as the rate of solubility in liquids, the rheological properties, and the clinical efficacy in pharmaceutical preparations, each of which is in itself a subject for research.

In the following sections of the paper, a brief description is given of the above fundamental properties.

The Size of an Individual Particle

An irregularly shaped particle has no unique dimension and its size can only be expressed in terms of the diameter of a sphere that is equivalent to the particle with a regard to some stated property. Such equivalent spheres are those which:

(i) have the same projected area as the particle when viewed in a direction perpendicular to the plane of greatest stability, symbol d_a;

(ii) have the same volume as the particle, symbol d_v;

(iii) have the same surface area as the particle, symbol d_s;

(iv) have the same free-falling velocity in a fluid as the particle, symbol d_f. (If the diameter/velocity relationship follows Stokes's Law, the symbol d_{st} is used.)

(v) correspond to a square aperture of side A through which the particles will just pass.

These equivalent diameters are based on different properties of the particle, hence their numerical values can only be identical for spherical particles and the divergencies will increase with greater irregularity in shape.

The various methods of particle size determination measure different equivalent diameters, for example the microscope method of measurement gives the equivalent diameter (i) above, sedimentation and elutriation analyses are expressed in terms of the diameter (iv) above whilst sieve analyses are in terms of (v) above. Consequently it is essential when reporting on a sizing analysis to specify the method used, since this can affect appreciably the numerical values obtained. The general relationship

between these various equivalent sizes can be determined experimentally, or calculated if the shape characteristics of the particles are known and are expressed in a numerical manner. Such relationships are discussed in greater detail later in this paper, though Fig. 1 shows graphically some of these equivalent diameters.

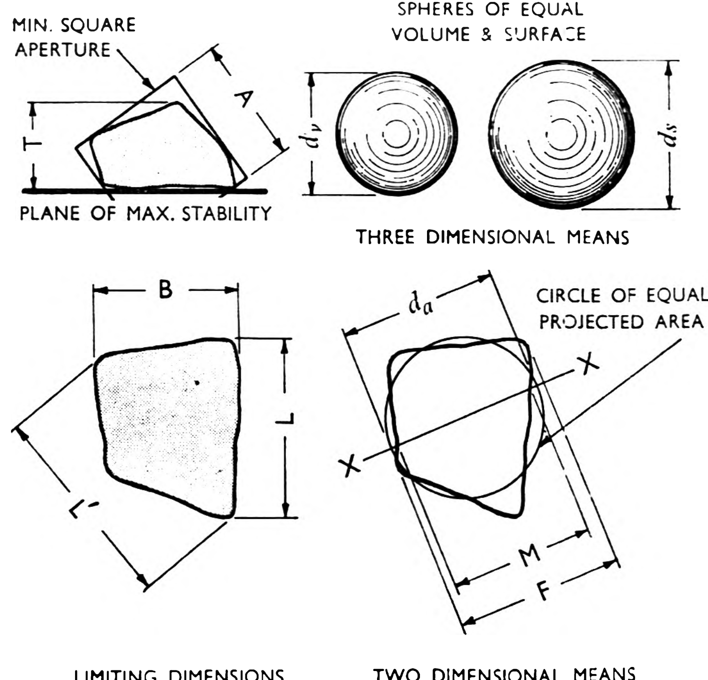

FIG 1. Equivalent particle diameters.

The Mean Size of a System of Particles

A system of particles is characterised by the properties of total number, length, surface area, and volume (weight, if density is uniform) of particles. If a system of non-uniformly sized particles is to be represented by a system of uniformly sized particles having the same shape (and density), the two systems can be equivalent as regards two, but only two, of the above properties. The size of the particles in the uniform system is then the mean size or mean diameter of the non-uniform system with respect to these two properties.

Mathematical expressions for these mean diameters can be derived if the complete size range of the particles is subdivided mathematically into a number of small groupings each of size range δx and with assumed uniform diameters of x_1, x_2, . . . The symbol x is used for diameter because the method of measurement for individual particles is not specified, and all particles are assumed to have the same shape. Let the numbers of particles in these groupings be δN_1, δN_2, . . . respectively. Then the aggregate length, surface area, and volume of the particles in

each grouping are represented by the expressions $x_1\delta N$, $x_1^2\delta N$, and $x_1^3\delta N$, respectively, and the totals for the whole of the powder by the summations $(x_1\delta N_1 + x_2\delta N_2 + \ldots) = \Sigma x\delta N$, and similarly for terms with higher powers of x. The expression $\Sigma x^3\delta N$ may be replaced by $\Sigma\delta V$, and the expression $\Sigma x^2\delta N$ by $\Sigma\delta V/x$, where δV represents the volume of particles in a grouping, or the weight if the density is assumed to be uniform for all particles. Table I shows in summarised form the mathematical expressions and the nomenclature for the various mean diameters.

TABLE I

DERIVATION OF MEAN DIAMETERS

Nomenclature and properties concerned	Expressions for means	Symbols
Number length mean diameter	$\Sigma x\delta N/\Sigma\delta N$	x_{nl}
Number surface mean diameter	$\sqrt{\Sigma x^2\delta N/\Sigma\delta N}$	x_{ns}
Number volume mean diameter	$\sqrt[3]{\Sigma x^3\delta N/\Sigma\delta N}$	x_{nv}
Length surface mean diameter	$\Sigma x^2\delta N/\Sigma x\delta N$	x_{ls}
Surface volume mean diameter	$\Sigma x^3\delta N/\Sigma x^2\delta N = \Sigma\delta V/\Sigma\delta V/x$	x_{sv}
Volume moment mean diameter	$\Sigma x^4\delta N/\Sigma x^3\delta N = \Sigma x\delta V/\Sigma\delta V$	x_{vm}
Weight moment mean diameter	or $\Sigma x\delta W/\Sigma\delta W$	x_{wm}

If this notation is combined with that described above for the different methods of measuring individual particles, then the resultant symbol defines completely the method of measurement and the properties represented by the mean diameter. For example, the result of measuring particles by means of the optical microscope and counting the number of particles corresponding to various size ranges would be expressed as $d_{a,\ nl}$. The surface volume diameter calculated from the results of a sedimentation analysis by means of Stokes's equation would be $d_{st,\ sv}$. The average value of the cumulative undersize weight percentage curve obtained by sieving, plotted against sieve aperture, would be A_{vm} or A_{wm}.

Although the system of nomenclature described above may seem somewhat complex the Author is convinced from many years of experience that it is the only system that will provide a unique definition of particle properties.

Particle Shape

This property has especial importance in connection with pharmaceutical products since it affects the surface area, bulk density, and other characteristics of the powder. Various types of particle shape have been defined in B.S. 2955 as Acicular, Angular, Crystalline, Dendritic, Fibrous, Flaky, Granular, Irregular, Nodular, Spherical. Such descriptions, however, are inadequate for the purposes of calculating particle properties which incorporate the effect of shape.

The surface area of a particle is proportional to the square of some characteristic dimension and the volume of the particle to the cube of this dimension; the constants of proportionality depend upon the dimension chosen to characterise the particle and the projected area diameter is

used for this purpose throughout the following discussion. Hence it can be stated that:

$$\text{Surface of particle} = fd_a^2 = \pi d_s^2 \quad .. \quad .. \quad (1)$$

$$\text{Volume of particle} = kd_a^3 = \pi d_v^3/6 \quad .. \quad .. \quad (2)$$

where f is the surface coefficient and k is the volume coefficient. These equations also enable the relationship between d_a, d_s, and d_v to be calculated.

The volume coefficient k may be determined from a knowledge of the number, mean size, weight, and density of the particles composing a fraction graded between close size limits. It is, however, more difficult to determine by measurement the external surface area of small particles, but the surface coefficient f may be determined by geometrical analogy from measurements made on larger particles. The general term "shape" comprises a combination of the geometrical form of the particle and the proportions of the particle. Thus one can visualise that in the special case of particles that are equi-dimensional, i.e., for which the limiting dimensions, length, breadth and thickness, are equal, the volume coefficient k has a unique value, termed k_e, which is solely a function of the geometrical form. The following equation then relates the coefficients k and k_e according to the proportions of the particle:

$$k = k_e/m\sqrt{n} \quad .. \quad .. \quad .. \quad .. \quad (3)$$

where m is the ratio of breadth to thickness and n is the ratio of length to breadth.

An experimental investigation (Heywood, 1954) on the geometrical properties of large particles led to the derivation of the following relationship between f and k_e:

$$f = 1{\cdot}57 + C\left(\frac{k_e}{m}\right)^{4/3}\left(\frac{n+1}{n}\right) \quad .. \quad .. \quad (4)$$

in which C is a coefficient depending upon geometrical form and other symbols are as defined above.

TABLE II

SHAPE COEFFICIENTS FOR EQUIDIMENSIONAL PARTICLES

Shape group	k_e	C	$Ck_e^{4/3}$
Geometrical forms:			
Tetrahedral	0·328	4·36	0·986
Cubical	0·696	2·55	1·571
Spherical..	0·524	1·86	0·785
Approximate forms:			
Angular: Tetrahedral	0·38	3·3	0·91
Prismoidal	0·47	3·0	1·10
Sub-angular	0·51	2·6	1·06
Rounded..	0·54	2·1	0·92

Table II shows the values of the coefficients k_e and C for certain geometrical forms and also for irregular particles which have been divided into four shape groups, for each of which these coefficients have been determined experimentally.

Using these coefficients, it is possible by making a visual assessment of the particle shape and proportions through the microscope, to calculate with reasonable accuracy the appropriate values of the coefficients f and k.

Density

This property is needed to relate the volumes and weights of particles. There are, however, complexities in the determination due to the fact that many materials contain pores which may be sealed or closed and so do not communicate with the external surface, or alternatively are open, and connected to the surface. Hence there are three values for the density of a particle which have been defined in B.S. 2955 in the following manner:

True density: Mass of a particle divided by the volume of the particle excluding open and closed pores.

Apparent particle density: Mass of a particle divided by the volume of the particle excluding open pores but including closed pores.

Effective particle density: Mass of a particle divided by the volume of the particle including open and closed pores.

These densities will be designated here by the symbols σ, σ_a and σ_e respectively. True density, σ, refers to the density of the material composing the particle, irrespective of the structure. Apparent density, σ_a would be used if the particle was immersed in a fluid that penetrated the open pores, e.g., in sedimentation calculations. The effective density σ_e would apply when the external surface was regarded as the boundary of the particle or when the ambient fluid was unable to penetrate the open pores.

Methods for determining particle density are described in B.S. 3483.

Surface Structure and Specific Surface

The permeable characteristics of particles are well known and the surface structure of many materials may contain sub-microscopical fissures or pores. The versatile scientist Hooke was aware in 1665 of the porous structure of charcoal, and indeed made an estimate of 5,725,350 pores in a circular area 1 inch in diameter! An interesting analogy suggested by Sir Eric Rideal (1934) was the area of a ploughed field. The projected surface of such a field as shown on a map would be less than the external surface allowing for the furrows, whilst the whole surface accessible to the air through the pores of the surface could be one hundred times as great as the external surface.

The three methods for determining the specific surface of a powder are:

(i) By calculation from the sizing analysis, and including the effect of the shape coefficients defined above. The following equation can be derived to express this factor:

$$S_{cal} = \frac{f}{\sigma_e k} \cdot \frac{\Sigma x^2 \delta N}{\Sigma x^3 \delta N}$$

$$= \frac{f}{\sigma_e k} \cdot \frac{1}{x_{sv}} = \frac{f}{\sigma_e k} \cdot \frac{10^4}{d_{a,sv}} \text{ cm.}^2/\text{g.} \quad \ldots \quad \ldots \quad (5)$$

where $d_{a,\,sv}$ is the surface volume mean diameter in microns, based on projected area measurements and σ_e is the effective density.

Such a calculation gives the minimum value of specific surface; the result is dependent on a precise knowledge of the surface volume mean diameter for the particles, which is not easy to determine accurately for very fine powders. Consequently this method should only be applied to relatively coarse powders and it is preferable to determine the surface experimentally by one of the following procedures.

(ii) Surface determination by permeability measurements. There is a mathematical relationship between the resistance to flow of a fluid through a packed bed of particles, the porosity of the bed and the surface area of the particles in contact with the fluid in flow. Liquids or gases may be used for the measurement, though air is the most frequently used medium in practice. Several designs of suitable equipment have been described in publications (Lea and Nurse, 1947; Rigden, 1943, 1947), one of which has been standardised for the testing of Portland Cement. Specific surface measurements made by the permeability method will include the effect of minute surface irregularities, but not the surface of the sub-microscopical pores which are inaccessible to the fluid flow; hence such values are usually slightly greater than those calculated from the sizing analysis.

(iii) Monomolecular layer adsorption can be used to determine directly the specific surfaces of very fine powders (Joy, 1953). The magnitude of the surface determination will depend on the extent to which the adsorbed molecules can penetrate the submicroscopical fissures; thus if relatively large dye molecules dispersed in a liquid are used for the determination, the surface area will be less than if gas molecules are used. The equipment for determination of specific surface by gas adsorption is fairly elaborate, and the procedure has not yet been standardised. It is possible that eventually some simplified methods may be developed for routine testing. The ratio of surface determined by adsorption to that determined by permeability is often found to be of the order of 3:1, and may be as high as 10:1.

Bulk Density of Dry Powders

A study of the classical systems of packing for spheres of uniform size has shown that the porosity, or void space, varies from 48 per cent for cubical packing to 26 per cent for rhombohedral packing, which is the irreducible minimum for such systems (Graton and Fraser, 1935). Furthermore, the voidage or porosity of such systems is independent of the size of the spheres. Practical conditions differ greatly from these simple classical systems, in that the particle size is not uniform, the particle shape may diverge from the spherical, and fine particles can adhere together forming loose chains or bridges with a high voidage. Although smaller particles can fit within the interstices between large particles, thus decreasing the voidage, in fact, the looser packing of the finer particles may nullify this effect, and the resulting voidage for mixed sizes may be greater than the theoretical for closely packed uniform spheres. Indeed, there is

approximate proportionality between specific surface and voidage in a dry powder, and the latter has been proposed as a rough measure of specific surface (Heywood, 1946).

Increased packing densities may be obtained by mixing powders in the wet state, whereby aggregates are dispersed, but this may not be feasible for some types of powder. The greatest density with dry packing is probably attained when a fine powder is mixed with a relatively coarse powder which is closely sized, but there should be a gap between the two powder sizes which should have a ratio of approximately 6 : 1. A paper on this subject led to the following two generalised conclusions:

(i) With relatively large particles, where cohesion effects are negligible and the accidental formation of arches or vaults is avoided, the porosity is decreased when the particles extend over a wide range of sizes.

(ii) With powders consisting of fine particles relatively large void spaces are formed by arching of the particles and by cohesion into chain formations. These effects increase the porosity and usually more than nullify the effect of particle variation, so that fine powders may have very high porosities.

The above features are illustrated by the following Figures. The arching of the particles, in this case steel ball bearings poured between two glass plates, is well shown by Fig. 2 (from Brown and Hawksley,

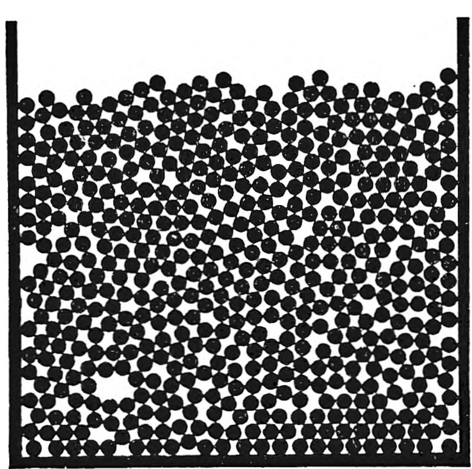

FIG. 2. Packing of $\frac{1}{4}$ inch ball bearings between glass plates, showing stable arches and high porosity at the walls of the container. (*From* Brown and Hawksley, 1945, *Coal Res.*, 143, with permission).

1945). The effect of the wall of the vessel in promoting large void areas is noticeable. The structure of very fine powders, such as carbon black and zinc oxide smoke is shown by the electron microscope photographs in Fig. 3. The voidage or porosity of such systems may be 96 to 98 per cent (Heywood, 1946). As examples of the porosity of ground minerals,

a limestone of which 46·5 per cent passed a 200 mesh B.S. sieve had a porosity when loosely poured of 51·7 per cent, but when ground to 93·7 per cent passing 200 mesh B.S. sieve the porosity was 71·0 per cent. A further study of the subject may be made from the references quoted at the end of the paper (Graton and Fraser, 1935; Melmore 1942; Heywood, 1946).

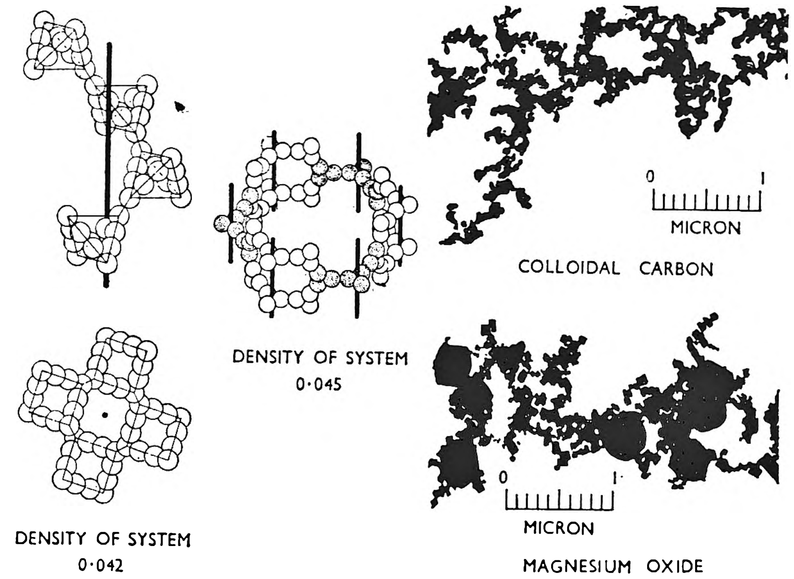

FIG. 3. Open packings of low density and electron microscope photographs of small particles. (*From* Heywood, 1946).

PROCEDURES FOR PARTICLE SIZE ANALYSIS

There is no single method of size analysis which can be used to cover the whole range of particle size concerned in industrial practice. Sieving procedures may be used for particles that are larger than 60 μ and below this size there is a variety of sub-sieve size analysis procedures using different principles of operation. In nearly all of these, however, the particle size concentration relationship is determined indirectly, such as by measuring free-falling velocity in a fluid, by the absorption of electro-magnetic radiation (light, X-ray, β or γ radiation), or by means of some electrical property. The microscope is the nearest approach to a direct method of measurement but even in this case the particle is only seen as a projected image in one plane. The effect of these different methods of analysis on the numerical results has been referred to in the section on "Size of an Individual Particle" above.

During the last thirty years, many individual investigators have published the results of their researches on methods of sizing analysis and described the designs of equipment used. There is still, however, considerable confusion as to the best methods to employ and the procedures to be adopted. Co-operative research between laboratories and scientific

organisations is essential for further progress leading to standardisation of accepted methods.

The British Standards Institution first published in 1931 a specification B.S. 410 for the dimensions and tolerances of Test Sieves and B.S. 1796 on methods for the use of such sieves was issued in 1952. Specifications for methods of determination of the particle size of powders are now ready for publication as B.S. 3406 in the following sections: Part 1. Sub-division of gross sample down to 0·2 ml. Part 2. Liquid sedimentation methods. Part 3. Air Elutriation Methods. Part 4. Optical Microscope Method.

TABLE III

SCOPE OF METHODS OF PARTICLE SIZE ANALYSIS

Method of analysis	Lower size limit; microns	Equivalent diameter measured
Sieving:		
B.S. Sieves. Normal.	75	A
Finest in series.	45	
Electroformed holes.	20	
Microscope:		
Optical: theoretical limit of resolution.	0·2	d_a or d_s*
practical limit of measurement.	1·0	
Electron; normal.	0·01	
Elutriation:		
Gravitational: air or water.	5–10	d_{st} or d_f†
Centrifugal: air.	5	
water, hydraulic cyclone.	8	
Sedimentation:		
Gravitational; liquid. Particle concentration measured by differential manometer; pipette extraction; collection on balance or in tube; hydrometer or "divers"; absorption of light, X-Ray, β or γ radiation; radioactive emanation.	2	d_{st} or d_f†
Gravitational: air.	1	
Centrifugal: liquid. Particle concentration may be measured by most of methods used for gravitational settlement.	0·05	
Automatic counting:		
Scanning of microscope slide or photomicrograph, or flying spot microscope.	1	d_a or d_s†
Coulter Counter; change in electrical resistance as particle passes through orifice.	0·2	d_t

* The diameter d_a implies that the particle is observed perpendicularly to the plane of maximum stability. In the case of very fine particles observed through the optical microscope, and certainly in the case of the electron microscope, the deposit of particles may have a random distribution of orientation and the mean projected diameter measurement tends to the diameter of the sphere of equivalent surface, d_s (Pidgeon and Dodd, 1954). This follows from a theorem of Cauchy that the surface area of a particle is 4 times the mean random area of projection (Cauchy, 1841).

† If Stoke's Law is valid the symbol d_{st} is used; at particle sizes greater than this limit the appropriate free-falling velocity should be calculated as explained in the section "Motion of Particles in a Fluid," p. 8.

These specifications are necessarily restricted to well established methods of size analysis but the detailed instructions given should guide those unaccustomed to such work and greatly improve the comparative accuracy of the analytical results obtained at different laboratories.

The Society for Analytical Chemistry formed a Particle Size Analysis Sub-committee in March, 1961. A review issued in March, 1963 (*Analyst*, 1963) has classified the known existing methods for particle size analysis, with a brief description of the scope and procedure for each method.

The data has been extracted from published papers and is not intended to be critical, but has the object of assembling in one document an account of methods which have been devised for sizing analysis. The work will be carried to a further stage involving a detailed examination of a number of particle size analysis methods and will involve comparative experiments with the aid of all types of laboratories concerned in this subject. Table III shows briefly the scope of the various methods of size analysis and in the following sections of the Paper a short description is given of the salient features of these methods.

Sieving Procedures

Sieving is an important method of sizing, particularly for vegetable products and for the coarser organic preparations that cannot be sedimented in water. If conducted properly the analyses are accurate and reproducible, but if performed incorrectly the results can be very misleading. The sieving process consists of two stages, firstly the elimination of the fine dust particles which are much smaller than the mesh apertures, though this may be difficult if the powder is sticky, and secondly the elimination of the near mesh particles which will only just pass through the apertures. This latter process can never be complete and the end-point of the analysis should correspond to a defined rate of passage, say 0·1 per cent of the sample weight per min., though a defined time of sieving may be adopted for routine testing if this is known to be adequate (Heywood, 1938, 1945).

The weaving tolerances of wire sieves have been standardised in Great Britain by B.S. 410 and standard procedures for sieving are described in B.S. 1796. This specification should be consulted for full details, but the essential features for dry sieving are a rapid tapping and shaking motion combined with periodical cleaning of the sieves with a soft brush to clear the apertures of dust. Only when the fine dust has been eliminated does separation of the near mesh particles commence. The fine dust particles are more rapidly eliminated by wet sieving, i.e., washing the dust through the sieve with a fine jet of liquid. This can only be done if a suitable liquid is available that does not dissolve the powder. The residue on the first sieve should be dried after washing, and re-sieved in the dry state; this is essential since surface tension will hinder the wet near-mesh particles from passing the apertures.

Skilled operators can repeat analyses by hand-sieving with accuracy and speed, but sieve-shaking machines are now often used in laboratories for routine testing. These can be quite satisfactory provided the sieve cleaning process is performed at intervals, otherwise the apertures may become completely choked by a difficult powder. Visual observations will show a change to a granular appearance of the residue on the sieve after the fine dust has been eliminated. Particles having the form of thin flakes or long needles (acicular) are difficult to sieve to finality, since they will only pass the apertures when presented in a favourable position.

The method of reporting sieving results is a matter of importance if ambiguity is to be avoided. The term "on" a certain sieve should not be

used since this could mean the percentage weight retained between two sieves or the cumulative percentage weight of the sample which is larger than the sieve concerned. The report should state definitely that w per cent passes through sieve X and is retained on sieve Y, or that the cumulative percentage undersize through sieve Y is W per cent.

Simple methods of graphical expression of the characteristics of a powder are more easily interpreted and a plot of cumulative percentage undersize by weight against sieve aperture to a logarithmic scale fulfils most purposes. This system of plotting can also be used to show the combined results of a sieving analysis and a sub-sieve analysis, e.g., by sedimentation.

Microscopical Measurement

Direct measurement of particles by means of the optical microscope or by the electron microscope may be necessary under certain circumstances, such as when the sample available is very minute, but in general, the procedure is time consuming and needs great care and experience to obtain accurate results. Since for most powders the frequency of occurrence of particles increases rapidly as the size diminishes, it is necessary to measure very large numbers of particles to ensure a representative count. Even using the method devised by Fairs (1951) and described in detail in B.S. 3406 Part 4, by which group counting is used at three degrees of magnification, the work required is still formidable. Furthermore the results are obtained on a number frequency basis, and though these can be converted to a weight basis by calculation, the error may be considerable when one realizes that one particle 100 μ in diameter has the same weight as a million particles 1 μ diameter. The situation is different when the particle size range is small, or for blood counts which are fairly uniform, and may be improved in the future by the development of automatic electronic counting and sizing methods. Although the theoretical lower limit of resolution of the optical microscope is $0{\cdot}2\mu$, it is generally considered that measurements cannot be made with accuracy below 1 μ diameter, even with monochromatic light illumination.

The size of the particles is estimated by comparison with a series of circles on an eyepiece graticule which are superimposed on the microscope field of view. Statistical methods by which an intersept of the particle image is measured, such as the dimensions M and F in Fig. 1, are now obsolete.

Apart from size measurement, however, it is always advisable to examine a sample of powder through the optical microscope to determine the approximate size range, to assess the particle shape and to investigate the suitability of dispersing agents if a sedimentation analysis is intended.

The limit of resolution of the electron microscope is of the order $0{\cdot}01\ \mu$. Size measurements by this means suffer the same disadvantages as with the optical microscope but to an even more pronounced degree. However, much can be learned about the characteristics of the powder by such examination, particularly of the shape of the fine particles and whether these are single or composed of aggregates of even smaller particles.

Motion of Particles in a Fluid

Since the methods of size analysis described in the following two sections are based on the terminal velocity of a particle settling in a fluid, a brief account is given here of the factors on which this is dependent.

A particle settling under gravitational acceleration attains a terminal velocity at which the drag or resistance to motion balances the effective weight of the particle immersed in the fluid. If the fluid motion round the particle is laminar or streamline in character, then the relationship between the terminal velocity and the size is given by Stokes's Law, namely:

$$u_{st} = \frac{d_{st}^2(\sigma_a - \rho)}{18\,\eta} g \quad \ldots \quad \ldots \quad \ldots \quad (6)$$

Where u_{st} is the terminal velocity, σ_a the apparent particle density, ρ the density of the fluid, g the gravitational acceleration, η the absolute viscosity of the fluid and d_{st} the Stokes diameter of the particle, all factors in C.G.S. units.

However, above certain limiting particle sizes and velocities, turbulent flow round the particle involves a drag force additional to that due to viscous forces. The criterion of flow conditions is the dimensionless group termed the Reynolds Number, namely:

$$\mathrm{Re} = \frac{u_{st} d_{st} \rho}{\eta} \text{ or } \frac{u_f d_f \rho}{\eta} \quad \ldots \quad \ldots \quad \ldots \quad (7)$$

in which u_f and d_f refer to free falling velocities and diameters in the flow regime beyond the validity of Stokes's Law.

Stokes's Law cannot be used if the Reynolds Number exceeds 0·2 (Heywood, 1953) and the corresponding critical value of d_{st} is given by the following equation:

$$d_{st}^3 = \frac{3{\cdot}6\eta^2}{(\sigma_a - \rho)\rho g} \quad \ldots \quad \ldots \quad \ldots \quad (8)$$

If settlement is by centrifugal force, then the appropriate acceleration must be substituted for g in the above equation. The critical value of d_{st} is 65 μ for particles of density 2·7 g./cm.^{3} settling gravitationally in water at 15°, and 35 μ for the same particles settling in air. Hence it is improbable that many powdered materials used in pharmacy will be beyond the range of Stokes's Law. However, the correct free falling velocities for larger particles may be calculated by a method described in a publication by the writer (Heywood, 1962), or determined from tables given in Appendix A, B.S. 3406 Part 2 for water and Part 3 for air.

Elutriation

This is a process of grading particles by means of an upwardly moving current of fluid, normally water or air. It has the especial advantage of subdividing a powder into a number of closely sized fractions which may subsequently be examined for chemical or clinical properties. As a sizing procedure, the method is lengthy and would only be adopted when essential, such as in cases where the powder may not be wetted. The

Haultain, Roller, and Gonell type air elutriators are widely used in industry, the two latter being described with details for operation in B.S. 3406 Part 3. This specification also describes a miniature elutriator which can easily be constructed in a laboratory workshop.

Air elutriation under the action of centrifugal forces accelerates the procedure and can be accomplished by a proprietary machine termed the BAHCO Classifier (*Analyst*, 1963). Centrifugal force can also be used for water elutriation, and a specific design of hydraulic cyclone for this purpose is described by the reference given (Kelsall and McAdam, 1963).

Sedimentation Analyses

The normal procedure is to allow a homogeneous suspension of the particles in a liquid to settle under gravity, and to determine at suitable intervals of time from the commencement of setting either the relative concentration of particles at a known depth below the surface or the mean concentration from the surface level to a known depth (Heywood, 1953). The former is termed an incremental method, and the latter a cumulative method; in both cases the size of the particle is calculated by means of Stokes's Law from the known velocity of fall corresponding to the time of determining the concentration. The definition of particle size is thus the diameter of a sphere of equivalent terminal or free-falling velocity.

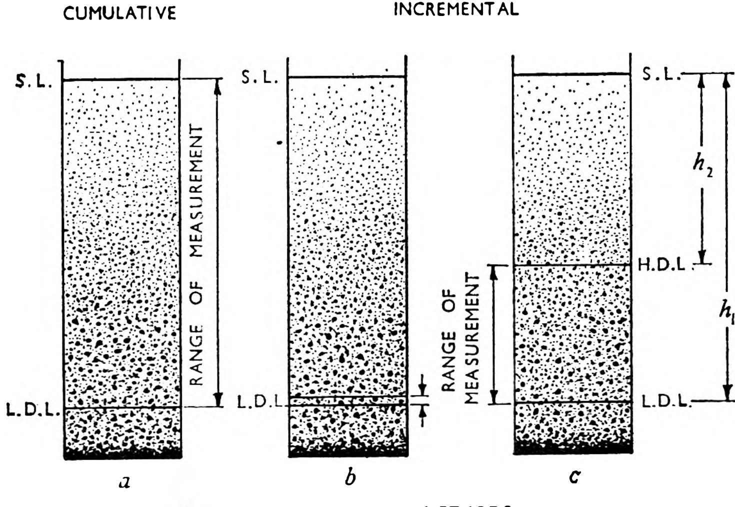

FIG. 4. Ranges of density measurement for sedimentation size-analysis methods. (*From* Heywood, 1945, *Trans. Inst. Min. Met.*, **15**, 373–390, with permission).

These procedures are illustrated in Fig. 4. If h_1 is the sedimentation height from the lower datum level to the surface level and h_2 the height from the upper datum level to the surface; then for cumulative methods $h_2 = 0$, whilst for incremental methods $h_2 = h_1$ and $h_1 - h_2 = 0$. This

condition, however, would involve sampling across a plane perpendicular to the sedimentation column, which is physically impossible. In some methods of test, i.e., by pipette extraction, the ratio $(h_1 - h_2)/h_1$ is about 4 per cent and the theoretical conditions are approximately attained, in others, i.e., the hydrometer method, $(h_1 - h_2)/h_1$ may approach 50 per cent and the method cannot be regarded as truly incremental. Cumulative methods may use the sedimentation balance, with or without automatic recording of the weight of sediment, or a tube at the base of the sedimentation column as a means of collecting the settled material.

Incremental methods give a direct measure of the particle concentration against equivalent size; in cumulative methods the experimentally determined concentration measurements at various times must be subjected to a differentiation process, which can be performed in a tabular manner or graphically, in order to obtain the relationship between particle concentration and equivalent size.

Concentration may be measured in a variety of ways. The simplest method is direct extraction of a small sample by means of a pipette, followed by evaporation of the liquid and weighing of the residual particles (Andreasen, 1928). Alternatives are density measurement by hydrometer or preferably by "divers," relatively small glass vessels adjusted to a known density which are in equilibrium with the suspension at a level where the density is the same (Berg, 1958; Jarrett and Heywood, 1954). If a piece of magnetic alloy is inserted in the diver its position may be indicated by a sensitive electronic indicator surrounding the sedimentation tube. Concentration may also be assessed by the absorption of electromagnetic radiation which may be visual light (Rose, 1950), X-rays, β- or γ-emanation (Connor and Hardwick, 1960). Such methods are particularly suited to the automatic recording of variation in concentration with time of settlement.

Effective dispersion of the particles is essential. A deflocculating agent should be added if water is used as the sedimenting liquid. Calgon is very effective for many powders, and sometimes a wetting agent is also required; pH value may need adjusting to an optimum value. In all cases, the sample should be dispersed by a high speed mechanical stirrer before pouring into the sedimentation vessel.

A proprietory apparatus, the Micromerograph, enables particles to be sedimented in an air column, the powder being dispersed at the top of the column and the rate of collection measured on a balance pan at the bottom of the column (Kubitschek, 1960).

As with elutriation, the application of centrifugal force greatly speeds the rate of settlement of particles of about 1 μ diameter, and is essential for particles below this size since the effect of Brownian motion begins to invalidate the sedimentation process. Descriptions of a number of designs for centrifugal sedimentation have been published, though not all of these have yet reached the stage of final development (*Analyst*, 1963). In all cases the size of the particle is calculated from the time of centrifuging and the rate and radius of rotation, but Stokes's Law ceases to be applicable at smaller sizes than for gravitational settlement. The

particle concentration may be measured by the same procedures as used for gravitational settlement.

Special mention should be made of the Coulter Counter which has recently been developed (*Analyst*, 1963). This is not a sedimentation procedure though the particles are dispersed in an electrolytic liquid. The very dilute suspension flows through an orifice which is part of an electrical circuit. As each particle passes through the orifice a change in electrical resistance occurs, which is a function of the volume of the particle. The electrical impulses are counted and monitered so that the relationship between number of particles and relative volume may be obtained directly. The significant particle size is the diameter of the sphere of equivalent volume, d_v, and the instrument is unique in this respect.

Equivalent Diameters and Comparative Surface Measurements

The relationship between equivalent diameters of particles as defined in the Section on size of an Individual Particle, p. 2, cannot be expressed in a simple numerical manner for all types of particle. Relative values for angular particles which are neither unduly flaky nor acicular have been quoted in B.S. 3406 as follows:

Projected area	Sieve	Stokes diam.
1·4	1	0·94

Individual experiments must however be made if accurate ratios for specific materials are required. This can be done by using two respective methods on the same powder over a range of overlap, e.g., by sieving or sedimentation, so that the correlation factor can be determined.

Specific surface determinations show an even greater variance according to method. Haynes (1961) quotes specific surfaces of clays determined by BET adsorption of 3 to 5 times the values calculated from sedimentation analyses. Adsorption of air or methylene blue gave values of the same order as the BET method, though greater in some cases. The BET values were 5 to 10 times greater than determinations by the air permeability method, though this latter method is not very suitable for clays.

Lea and Nurse (1947) also give some relative specific surface values. In general, nitrogen adsorption gave values 2 to 10 times the calculated external surface and about 2 to 6 times the values by air permeability. Air permeability values exceeded those obtained by light extinction by about 50 per cent. Thus surface values obtained by different methods cannot be compared directly and the factors can vary greatly for different materials. There is much scope for research in the future to extend knowledge on the many variables concerned.

SPECIFIC PROBLEMS IN PHARMACY

The foregoing sections have dealt with fundamental problems of particle technology in general. The writer does not claim any detailed knowledge of pharmacy, but there are a few features concerning this field

on which comment could be made and attention drawn to certain instances where special treatment may be needed because of unusual characteristics of the products concerned.

Sieves should be used for the analysis of the coarser fractions of milled vegetable products, followed by air elutriation or air sedimentation if sub-sieve analysis is required, since with such materials wet methods would be inappropriate. Many pharmaceutical products are soluble in water and hence any sub-sieve analysis must be performed in a liquid to which they are inert, though this should not present any fundamental difficulty. Again, many of the crystalline materials may have a lamina or acicular shape which will complicate the sizing procedure.

Of the many properties mentioned by K. A. Lees in his conjoint paper, that of specific surface appears to be paramount. Since in general the specific surface varies inversely as particle size, this latter is a dominant factor in the properties of pharmaceutical materials.

Control of particle size may be accomplished by:

(i) reduction from a larger size down to the size required by mechanical milling

or (ii) growth from molecular size by controlled crystallisation or precipitation up to the desired size.

Breakdown by mechanical milling will unavoidably produce a hetero-sized distribution ranging from a minimum to the maximum particle size. When a brittle particle is fractured it breaks down to a spectrum of sizes and in general the frequency of particle occurrence is inversely proportional to the particle size. Some control in the final size range may be accomplished by a supplementary process, e.g., air elutriation, or classification in a hydraulic cyclone, but it is virtually impossible to obtain a monosized system of particles on an industrial scale by grinding methods. Controlled crystallisation or precipitation may however be used to produce a powder which does approximate to a mono-sized distribution. Many such powders consist of extremely fine particles for the sizing of which some centrifugal method of sedimentation would be essential.

Future Developments in sizing Analyses

The equipment for sizing analysis, particularly that described by the British Standards Institution specifications, is necessarily such as can be constructed in a well equipped laboratory workshop and generally requires manual control and operation. It is inevitable that the development of particle size analysis equipment will follow the general trend of scientific progress in extending the use of electronic systems of measurement, together with automatic recording and control (Connor, 1963; Lambert, 1963). Some indication has been given in earlier sections of the paper of the way in which such developments are taking place but a word of warning should be interpolated. Too often is electronics regarded as a substitute for experience, but in fact the experienced operator is still invaluable and with simple equipment can obtain analyses that are

reliable even if more time consuming. This statement is not intended to deprecate the labour saving value of electronic equipment, but merely to emphasise that some alternative system of checking results is essential.

Particle size analysis as a process, is merely a means to an end, the end being the application of the powder to some industrial or scientific purpose; in so far as this Conference is concerned, the field of pharmaceutical applications. These purposes have been outlined in the paper by K. A. Lees, and it is apparent that the closest co-operation is needed between laboratory research and clinical experience to solve the many problems involved. Such clinical tests are difficult to make and lengthy in time, but without them we may be in danger of amassing much fundamental knowledge which cannot be applied in practice.

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DISCUSSION

Caking and aggregation should be avoided in fine particle systems. How aggregation affected solution rate largely depended upon the nature of the drug itself. As the particle dissolved, molecules left the solid phase and concentrated in a saturated layer on the surface of the particle; solution was the dissipation of that layer throughout the bulk of the liquid, and the speed at which this occurred governed further solution of the particle. It was agreed that a study of fine particles might be useful in relation to prolonging as well as increasing the rate of absorption of drug. Separation of powder from granules could not be avoided where there was freedom of motion but it might be reduced by increasing the adhesion of the particles by coating them. The problem of separation of powders during storage was most serious when there was a small proportion of active ingredient, but this could be overcome by ensuring the absorption of the active ingredient on a diluent with a suitable surface area. The shape of the insoluble particles was the predominating factor governing the viscosity of suspensions. In producing particles to a certain size range it was often better to start with a large particle and mill it down, destroying the crystal shape, rather than to attempt to crystallise directly to a mean particle size. The stability of a drug could be affected by its particle size and a reduction in size often increased the breakdown of dry particles. Contrary to the general rule, a decrease in the particle size of aspirin anhydride resulted, in the absence of oxidative mechanisms, in an increase in stability. This was due to a release of mother liquor. The presence of grit particles in a preparation might conveniently be detected by a conical gauge apparatus utilised in the paint industry. It was suggested that surface energy might be the factor involved in the relationship between particle size and solubility. Protective colloids such as methylcellulose could minimise the tendency for crystal habit to change, but in some circumstances a colloid might cause aggregation. The danger of attempting to represent the characteristics of a complex powder by means of a one-point measurement was stressed.

CONFERENCE LECTURE

TOXIC HAZARDS FROM DRUGS

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Toxic hazards that might accompany the use of drugs have always been recognised by some, but it is probable that many of those who market and prescribe drugs largely discount the hazards and act on the assumption that existing practices and methods of control protect them and also those who receive the drugs.

In 1937 an enterprising chemist found that the problems presented by the insolubility of the new "wonder drug" sulphanilamide could be overcome by using diethylene glycol as a solvent. The resulting elixir, because of this solvent, killed at least 70 people (see Report of A.M.A. Chemical Laboratory, 1937). A tightening of the controls of the marketing of drugs by the U.S. Government soon followed (Anon., 1938).

Recent tragedies in the United Kingdom and Western Europe arising from the effects of a "safe" sedative have led to a renewed awareness of danger and consequently a demand for action to prevent risks of further injury from the toxic effects of drugs.

Similar situations arise in other fields of human activity. Farmers and growers all over the world had fought a long, hard battle against a variety of pests with a few dangerous and many rather ineffectual chemical agents when DDT and BHC arrived on the scene at the end of the last war and scored an immediate success. These insecticides were closely followed by parathion which, used with the same carefree techniques that were adequate for the safe application of DDT, promptly caused a number of deaths. These tragedies aroused a sudden interest in dangers from the use of chemicals on crops. In this country enquiries and control measures, which included some legislation for the protection of operators, followed and these controls, despite continued anxiety expressed in some quarters, have led to the safe use of pesticides in the United Kingdom.

Thirty years ago an industry might decide to use a new material in a chemical or manufacturing process without making any particular enquiries about its toxicity to mammals. When men or women exposed to the new material became ill or some even died, enquiries might then be made. Incidents in which more than a few people were involved led to an awakening to these new potential dangers. The whole position has now changed so that all responsible users of new industrial chemicals begin by seeking information about the possible hazards their use may present to those who may be exposed to them.

Except among a few enlightened people the atmosphere of many of our cities had been accepted as a natural accompaniment of urban life and the fogs of London were even a subject of humour or hallowed

tradition. A few catastrophies elsewhere failed to shake this complacency until 1952 when a concatenation of circumstances led to the publicising of the heavy mortality among those exposed to a prolonged London fog (Anon., 1953).

Life on this planet has always been surrounded with threats in one form or another to its continued existence. Human achievement in all fields has depended upon some individuals pushing out into the unknown and taking risks. Progress cannot be achieved without a simultaneous acceptance of some hazards. Even stagnation is not necessarily free from danger.

For many centuries the physician was called upon to deal with human ailments with no more than a handful of drugs which possessed any therapeutic value and a number that were probably harmful as well as being ineffective. Now, thanks to the advances in chemistry, physiology and allied sciences, the physician has a plethora. Not only can they be used for the treatment of serious disease but others may ameliorate the minor disorders and discomforts which are increasingly conspicuous in modern life.

The rising standards of public health have rid communities of much illness, but such standards also demand more from those who are concerned with possible hazards to health in places of work, the general environment and in the home. Every proposed additive to human food is closely scrutinised lest its consumption might prove to be injurious. For some curious reason far more attention is now paid to synthetic chemicals despite the well known fact that all the most toxic substances are of natural origin.

The industrial medical officer has the services of the engineer and technologist to rid the factory of whatever he believes to be a toxic contaminant. Thus he may be left free to deal with complaints arising from the discomforts produced by heat, noise or monotony.

In the same way, the doctor at home is called upon to ameliorate a host of minor complaints. He naturally turns to the armamentarium of drugs so temptingly put in his way by an enterprising pharmaceutical industry and freely provided by a benevolent health service.

Toxic hazards arising from the administration of drugs and their possible control should therefore be examined in the context of the situation in which they occur and useful comparisons can be made with toxic hazards met with under other circumstances. The object of this examination must be to make recommendations for actions that will effectively reduce the risks accompanying the proper use of drugs. It should be accepted at the outset that nothing will eliminate all risks, while allowing the effective use of drugs. The ineffective use of drugs may be a potent source of trouble but it cannot be considered here.

As in many other situations, it is probably too much to ask that the problem of the toxic hazards from the use of drugs should be viewed in perspective. The tremendous publicity and expensive advertising in all the media of communication aimed at reducing road deaths that have remained almost stationary at between 5,000 and 6,000 annually for 20

years may be compared to the efforts made to reduce cigarette smoking. This has caused deaths from lung cancer to rise fivefold from 5,000 to 25,000 per annum, in the same period. This disease kills more people in 1 year than motor traffic does in 4 years.

On the farms of the United Kingdom, tractors and bulls kill more people in 6 months than all the so-called dangerous pesticides have done in the 15 years of their wide scale use. Yet compare the number of committees considering each of these hazards. Many dangerous chemicals are used in industry but deaths and injury are mainly caused by machinery.

No one doubts that lives have been saved or health and activity restored by the use of new drugs which may also have injured a few of those receiving them. Those who die from diseases no one can treat die "natural" deaths. Deaths following the use of drugs given in good faith are liable to lead to enquiries that would have been avoided had no drug been given. The toxic hazards from drugs or other chemical substances must be considered from two general standpoints.

The *toxicity* of a compound is its capacity to cause injury while the *hazards* represents the probability that it will do so. It is very important to separate these two aspects of the problem when considering ways of ensuring the safe use of drugs.

Toxicity

The toxic effect of a drug may be direct and obvious and due to an excessive pharmacological action caused by an overdose or by the undue sensitivity of the individual receiving the drug.

The toxic effect may be an unexpected side-effect such as liver necrosis following an anaesthetic or aplastic anaemia from one among the many antibiotics. It is perhaps important to beware of the toxic effect which is not due to the drug. For example the widespread incidence of hepatitis once believed to be due to the drug Salvarsan was later shown to be due to the virus of infective hepatitis transmitted from patient to patient in inadequately sterilised syringes (Bigger, 1943).

However, the genuine and completely unexpected side-effect of a drug is the problem in toxicity that rightly excites most attention. The main problem in controlling hazards from drugs centres on the satisfactory recognition of such side-effects at an early stage in their incidence.

Two special toxic effects must be briefly mentioned. The first arises from the sensitisation of a proportion of the individuals who receive a drug. Individual sensitisation can occur to almost anything whether naturally occurring or synthetic. It is therefore not a problem confined to the distribution and use of drugs and such is the range of individuality in this respect that the world cannot be made comfortable for everyone prone to show this reaction.

The other special toxic effect is addiction. While this has a strictly pharmacological aspect in that some drugs are particularly prone to produce addiction in any individual receiving repeated doses of them, there is also a strong human element in other cases where addiction to

some unusual drugs may occur. The subject is a complex one and may, in some cases, be related in unexpected ways to developments in modern civilisation (see below).

Hazards

The hazards from drugs will be related to the numbers who receive the drug and the dose administered. This last point has been well emphasised in accounts of agranulocytosis and aplastic anaemia in patients receiving chloramphenicol. In many of these cases the amounts prescribed greatly exceed those recommended (Hodgkinson, 1954). The toxic hazards may be enhanced because those who receive drugs are not healthy. But against the hazards which may be attributed directly to the drug must always be set those which would follow were the drug to be withheld, or alternative treatment applied. Thus a drug given to suppress thyroid function may carry some risk of inducing a serious or fatal agranulocytosis whereas an alternative treatment would be surgical removal of the gland or the injection of radioactive iodine which also carry a certain risk. However, in situations like this where the disease and its sequelae are well known and each form of treatment has been subject to many separate analyses by experts in different hospitals, the related hazards are well understood and can be accepted by all rational people. Where a drug is administered to produce an effect whose benefits are completely uncertain such as a drug to lower blood cholesterol and where administration is likely to be very prolonged and under very marginal medical supervision, the significance of any toxic side effect assumes very different proportions.

Finally, the real hazards will be appreciated only if any adverse effects of the drugs are detected and their significance recognised. The hazards will also be related directly to the nature of the toxic effect which may be either rapidly fatal, or rapidly reversible or leave the patient permanently injured. In this last respect the hazards from occupational and environmental poisons can be strictly compared with those from drugs. But considerations of hazards in relation to benefits is almost confined to the field of drugs. Thus an individual should not be expected to run a serious risk of poisoning when applying a pesticide that will mean others will benefit from the better crop resulting from the use of the pesticide. Nor should a factory worker be exposed to a greater risk because a commercial enterprise can be made more competitive by the use of a cheaper but more dangerous solvent.

It is worth bearing in mind that had the use of thalidomide saved pregnant women from a serious or fatal disease, the ultimate birth of deformed offspring would have been accepted as a price that had to be paid for the saving of the valuable lives of their mothers. Fortunately women in the early stages of pregnancy are usually in excellent health despite the discomfort of "morning sickness" and to attempt to alleviate this at the possible expense of the foetus seems inexcusable—in retrospect. It is perhaps salutory to consider the ways in which effects of the tragedy of thalidomide may be viewed. "The thalidomide tragedy did not

directly affect your Company but one of our most widely prescribed products . . . is for use in pregnancy sickness. Inevitably, since the discovery of the wholly unforeseen risks attendant on the use of thalidomide doctors have become hesitant about prescribing any drugs during the early stages of pregnancy" (Eley, 1963). It is to be hoped that commercial enterprises will follow the doctors by practising a similar hesitation in recommending drugs for this physiological condition.

The Study of Toxicity

It is now appropriate to consider in more detail what we can know about the toxicity of a compound. Toxicity is not a property capable of precise measurement like boiling point or molecular weight but the toxic effects of a drug will be related to the dose, frequency and route of administration and nature of the injury produced. Special toxic effects such as sensitisation may be brought out by topical applications of drugs like penicillin in doses far below those causing any general toxic effects.

The toxic side-effects of drugs are, or should be, the subject of special investigations on laboratory animals. Various recommendations for the type of investigation that should be made have been put forward (Lehman and others, 1959; Paget and Barnes, 1963), but their value depends far more on the quality of the man making the investigation than on the detail with which suggested procedures are laid down by experts or by committees.

The simplest early tests are made to discover the relation of the lethal dose to the supposed therapeutic dose and to decide whether death is due to the pharmacological action of the drug or to some side-effect perhaps superimposed upon the main action. Tests on different species will determine whether the effect is general or limited to a few species or highly varied in the different species making the necessary extrapolation of man correspondingly more difficult. What is not always realised is that the value of all subsequent investigations on the nature of such a suspected toxic action will depend upon the skill with which the investigator analyses the original observations so as to make his subsequent experiments add to his preliminary information. The extension of toxicity testing is not to make the world safe for rats or dogs but to extract information from the reactions of these species that will make the world safer for man.

Many drugs call for repeated administration and therefore toxicity tests must include animals similarly treated. Non-toxic doses will often be those showing no effect on the growth, food intake and general condition of young animals. Special tests of function related to the action of the drug may be included and the fate of the drug should be studied so that this may then be compared with its metabolism in the first patients to receive it. Very often the final arbiter in deciding that the repeated administration of a drug was innocuous to animals will be the pathologist called upon to look at the tissues of those killed at the end of the experiments. In order that the pathologist shall be able to recognise

small degrees of abnormality in the material which he is studying it is essential that his control animals be healthy. For this reason they should be comparatively young and senescent changes must be absent. They should also be, as far as possible, free from changes due to the common infections widespread in so many animal houses. This means that tests in which the drug is administered repeatedly should not run for more than a few months in small animals and should be carried out on specific pathogen-free stock. It seems to be the consensus of opinion (Dr. G. E. Paget, personal communication) that no new toxic effects will be discovered if a drug is administered for longer than 3–6 months provided that the short term studies have been adequately performed. Tests of much longer duration have to be carried out to determine whether a drug has a carcinogenic action on the test animals but the pathologists' problem is not so difficult when this involves the recognition of the existence of a tumour. Far too little attention has been paid to the importance of pathological work on laboratory animals and to the training of people for its performance. Whereas 50 years ago morbid anatomy and histology were often the end-point in pathology, today histology should more often be the starting point of further investigations because sensitive techniques have given evidence of slight tissue changes that may herald more significant ones if the administration of a drug is either increased or prolonged.

A comprehensive series of toxicity tests can be concluded with the following sort of information. The proposed drug produces liver necrosis or kidney damage or severe failure of growth or food intake in doses close to those likely to be prescribed. The compound is then rejected as a potential therapeutic agent unless it is likely to be valuable in an otherwise certainly lethal disease. Possibly it will be found that repeated doses produces a certain incidence of tumours in the animals used in the toxicity tests. It will then be necessary to consider whether the drug is to be used by the young or the old, for serious or mild conditions and whether it is unique in its therapeutic effects or only an alternative to a drug already shown not to have these properties. It cannot of course be fairly compared to a well established drug that has never been tested by the same procedures to determine whether or not it has such properties. It is quite possible that one or more drugs now widely used and long considered safe might, if subjected to some of the procedures recommended for testing substances for carcinogenicity, produce tumours in laboratory animals.

These and many other bits of information may be learnt from well conducted toxicity trials on laboratory animals but however innocuous the drug may appear to be in the dose ranges that are to be recommended there is still no guarantee that (*a*) a certain number of people who receive the drug will not develop agranulocytosis; (*b*) no one will become very sensitive to the drug; (*c*) that if given to pregnant women the foetus may not be injured; (*d*) that some quite new effect will not first be seen in some human beings who receive the drug. A detailed analysis of the findings in rats, dogs and man has been made by Litchfield (1962) who

compared the reactions of 6 drugs of different pharmacological activity which had been tested for toxicity in dogs and rats and then given to at least 500 patients. From the animal tests it was possible to predict that certain toxic effects might be seen in man and 26 such predictions out of a possible 38 were correct. It was also possible to suggest what effects should not occur and out of 48 such possible instances 38 predictions were correct. This seems to be the only instance where an attempt has been made to find out how valuable tests on animals may be as a guide as to what may and what may not occur when a drug is given to man. Clearly the conclusions from this investigation are that tests on animals must continue to be the basis upon which first decisions about the safety or otherwise of a drug are based. Toxicity testing can reasonably ensure that the drug is unlikely to produce some well recognised toxic effect either generalised or confined to one organ in all the patients who receive the first doses to treat their ailments.

Assessing Hazards

If toxicity testing can give such a limited warranty of absolute safety it is then necessary to consider the hazards in more detail always remembering that there are other hazards that could arise if the drug was not given. Thus a new drug may relieve a serious disease but cause a certain number of casualties. Such an occasional effect is likely to be recognised early because the treated patients, suffering from a serious disease, will be under close medical surveillance. Similarly if a completely new compound is introduced into industry the medical officer responsible for the health of the exposed population will be on the alert for any new reports of illness. When a new pesticide is introduced into agriculture which by evidence of studies on animals appears likely to present a hazard then its introduction will be limited or the inspectors of the Ministry of Agriculture as well as the medical adviser of the manufacturer will be on the alert for reports of poisoning.

A drug, whether old or newly developed, may be found to relieve the symptoms of some common and harassing disease that is neither dangerous nor lethal. If the drug causes toxic side-effects then the speed with which these come to be recognised will depend very much on their nature. Thus a drug that relieved dyspepsia or the pain of peptic ulceration but caused peripheral neuritis in a significant number of those receiving it might soon be recognised as the probable cause of the new complaints. A drug that was found to give immediate relief to anxiety or neurosis might have to produce outstanding neurological or psychiatric side-effects before it became recognised as the cause of a new disease. For homely remedies the delay in recognising toxic effects from their administration may be very long indeed. In 1914 a disease of infants called acrodynia or "Pink Disease" was first described and it has been reported in Europe, America and Australia. Its aetiology remained a mystery until 34 years later when its possible association with mercury poisoning was put forward (Warkany and Hubbard, 1948). The mercury was in most cases undoubtedly that present in many infant teething

powders. A plea to make it illegal to add calomel to such preparations was still being made in 1954 (Dathan, 1954).

The problem of recognising toxic effects may face an industrial medical officer where a new process was introduced and within a short time a number of operatives applied for a transfer or left their employment. If the new chemical to which they were being exposed only caused rather general effects such as headache, fatigue, sleeplessness or anxiety, the operatives might not associate these disturbances with the introduction of a new solvent but attribute their complaints to some quite extraneous cause. Thus the hazards presented by a new toxic material may only be recognised early if the toxic effects are outstanding in their incidence or their manifestations. A new drug might induce severe sensitisation in 1 in 1,000 people who received it in the recommended doses. This might hardly impress itself on a practitioner with a total of 3,000 registered patients of whom he is unlikely to give one particular drug to more than a small fraction. The physician in an out-patient department of a large hospital might see several such cases but find it difficult to pin-point the cause of the illness to the use of a new drug of the use of which the patient might not be aware.

Other hazards may, of course, arise as the result of new techniques of promotion. Carbromal and bromvaletone had for many years been known to relatively few people as safe sedatives and they were present in a number of proprietary preparations available over the counter. A few years ago increased advertising including the use of television led to a greater number of people being introduced to these drugs and a few became addicted to them, sometimes with disastrous results.

Thus safe and useful drugs may prove to be a hazard for a few who discover them through popular advertising and do not take them under medical supervision (Seager and Foster, 1958; Copas, Kay and Longman, 1959). The question of the control of such drugs raises the problem as to whether this should be done solely in the interests of the unstable minority of the population (Glatt, 1959).

The fear that the widespread advertising on radio and television of the so-called safe sedatives containing phenacetin might lead to an increased incidence of renal damage has recently been published (Friend, 1963). So serious has the problem of renal damage due to phenacetin poisoning become in some countries that the drug is no longer available for purchase over the counter. The dependence which some acquire for the pharmacological action of ethanol is well known as a social problem but the hazards from other solvents such as trichloroethylene as drugs of addiction are not widely recognised and may prove fatal (James, 1963). That new uses may introduce new hazards was illustrated about 15 years ago when a drug discarded as dangerous found a new use as a weed killer in agriculture. Used without any particular care for long hours in hot weather to spray young corn, dinitro-*ortho*-cresol was responsible for a few cases of serious and even fatal poisoning among agricultural workers in the early years after its introduction (Hunter, 1950). Subsequently it proved possible to devise techniques by which it could be safely applied.

Control of Hazards

Hazards from all sorts of toxic chemicals depend upon how they are used. This could apply to solvents, pesticides or drugs. What are their physical and biological properties? How many people are exposed to them? What quantities do they receive? The industrial medical officer will examine the use of a new substance in the factory for which he is responsible. A new pesticide will not be used until the proposals for its application to seeds, crops or stored food have been scrutinised by expert committees. A similar screening procedure is promised for drugs. What might an enlightened Society do to protect its members from the introduction of drugs that could thereby put some or all of the members of that Society at some risk? Control could aim at four targets.

1. *Fewer new drugs.*

2. *The promulgation of new laws.* These would be designed to control the introduction of new drugs with the implication that only those of recognised value and with little or no associated hazard would be offered for sale or prescription.

3. *Better education.* This would be aimed at those people responsible for the administration and distribution of drugs so that only people in real need received drugs which were of recognised value and of proven safety.

4. *More research.* Support for research might make it possible to distinguish safe and dangerous drugs before they are given to large numbers of human beings.

No one would probably dispute that if all these steps were implemented in a general and enlightened way Society might be a safer place as far as risks from drugs were concerned but it is necessary to look a little more closely at each type of control as some favour one or more in preference to others. To each, serious drawbacks can be seen if they were to become the dominating means of control.

Fewer drugs. This may appear to be the simplest way of limiting hazards but if the emergence of new and more specific remedies is impeded this will mean that some patients will continue to receive larger doses of a less effective and possibly a more dangerous drug. Furthermore the full value of a new drug can never be assessed until it has been given to sick people and its effects observed. The reason that there are perhaps too many drugs available at present is that it is difficult to make out a case for discontinuing a drug that is not obviously better as a therapeutic weapon than another which has been long in use and shown to have a fairly well recognised margin of safety and incidence of side-effects. There is no case to be made out for restricting the number of drugs entering proper clinical trials, always provided that some satisfactory preclinical tests on animals have been carried out and adequately recorded. The clinical trial will tell the observers whether or not the new drug was superior to existing methods of treatment which in some cases will be the use of other drugs. Very few clinical trials reveal the full toxic potential of a new drug.

Even when the possibility of a toxic effect upon the bone marrow had been considered probable because of its chemical structure a very full trial of chloramphenicol "failed to disclose any evidence of this" (Smadel, 1949). Yet the wider use of this antibiotic has led to many cases of marrow damage being reported.

That there may be more drugs available than are really necessary to treat the diseases and illness encountered today is partly due to the difficulties of deciding whether significant differences do or do not exist between a number of different substances recommended for treatment of the same condition.

Some drugs linger on because they are believed to be valuable by those who use them. However such "clinical impressions" are notoriously difficult to measure or to assail. Perhaps it might be true to say that drugs are as reliable as those who prescribe them. No physician is perfect and neither is any drug. A careless or a non-observant physician is the equivalent of a potentially injurious drug. No label, "safe" or "dangerous", can be applied to a drug any more than it can be applied to a chemical used in industry. Safety depends in the ways in which substances are applied or prescribed. No case can therefore be made for restricting the numbers of new drugs *per se* but everything is to be said for the provision of better means to follow the use of new drugs and for the continued education of the users.

New Laws

Leglisation to control the introduction of new drugs has a good deal to be said for it but it must be remembered that the Law itself is poor protection against injury. It may exact penalties from those who are caught infringing its provisions but it is usually singularly ineffective in providing remedies for those who are injured. Laws may give the appearance of a safeguard but their existence does not deter the criminally minded. To some extent the existence of a law and the penal sanctions that accompany it may deter those who wish to indulge in doubtful practices. The section of the Pharmacy and Poisons Act which enables the Poisons Board to put certain drugs into Schedule IV (that is available on doctors' prescription only) has undoubtedly prevented much drug addiction and misuse of valuable therapeutic agents. So many new drugs are now pharmacologically active agents that it is questionable whether any new or worthwhile remedies should be available for the public to try for themselves. However some people clearly hope that new legislation will make it possible to prevent the introduction of drugs which subsequently prove so injurious that they have to be withdrawn. Unfortunately it is impossible to devise a set of tests to which a new drug must be submitted and the results of which will make it possible to say unequivocally that the drug is safe or dangerous. It is certain that a law could make it necessary that any new drug should be registered before it becomes available, particularly for prescription within the Health Service. Under such a law it could be stated that a Statutory Body would have to scrutinise evidence and state whether the drug had

been examined sufficiently comprehensively for both therapeutic value and adverse side-effects. However if a drug is passed by such a Body it must inevitably gain some hallmark of apparent safety. Those who press for legislation on this matter might well pause to consider whether more would not be achieved by enlisting voluntary co-operation. At least no drug need be made available under purely domestic rules of the Ministry of Health until the tests on animals to which it had been submitted had also been scrutinised by a committee appointed by the Ministry. If this Committee made an error in rejecting a drug of undoubted value, the drug might still find a place on the open market of private practice where the doctors and manufacturers shared the responsibility for any damage it might do to the patient. If it then proved to be unexpectedly safe or effective its further use could be reconsidered. Under a purely legal sanction errors of judgement might lead to the permanent exclusion of a potentially valuable material. It is necessary to face squarely the issue that tests on animals may lead to a safe drug being excluded or a dangerous drug accepted. It is therefore illogical to press for the rigorous application of toxicity tests on animals as a basis for the exclusion or acceptability of a new substance as a drug. No tests on animals can show that a drug is safe. These tests on animals may show that a drug has toxic properties which may either lead to its exclusion from further use or to its introduction with due care and watchfulness for such side-effects as have been observed in animals. It is still possible to buy ragwort from a herbalist although there is published work showing the poisonous action of this plant on livestock and the alkaloid present in ragwort will not only kill rats as a result of an acute liver necrosis within a few days but in smaller doses lead to their death from liver cancer 18 months later (Schoental and Magee, 1959). How can we logically accept tests on rats as a basis for accepting or rejecting drugs fresh from the synthetic chemists bench when we allow free sale, not even under medical control, of preparations which on the basis of studies on laboratory or domestic animals would never have been considered for sale as drugs? In this illogical Society must all the controls be placed on those who attempt to meet requirements by providing information while those who rely on ignorance and folk lore are allowed to distribute without any control whatsoever materials that are dangerous? If the recipients become ill and are finally obliged to seek real medical aid they rarely, if ever, admit their previous folly in consulting unqualified distributors of herbal remedies. If care is not exercised at this stage much effort will be spent in protecting the few from an occasional mishap occurring in the cause of a *bona fide* attempt to provide what is believed to be scientific treatment while others are exposed to the unscrupulous uncontrolled distribution of Nature's so-called remedies.

Education in the Use of Drugs

Patients receive drugs either because their medical attendants believe that the drugs will alleviate their illnesses or because the patients demand them. In this country it is unusual, though not unknown, for patients

to demand a specific drug. In some countries where popular journalism presents medicine and therapeutics in a way that all and sundry can recognise and remember the names of remedies, doctors may be put in a more difficult position. Sometimes the puzzled doctor may prescribe a simple and probably harmless drug for a week or two so that when he sees the patient again he can judge whether or not the signs and symptoms he first observed and whose significance to him appeared doubtful now point less ambiguously to the diagnosis. It is essential that a drug used in this way shall at least be completely harmless whatever be its therapeutic attributes. If the patient demands no more than a "bottle" as indeed they did 25 years ago, at least a harmless mixture could be prescribed. But if the patient demands specific treatment for his or her symptoms and even names the drug or type of drug required there is no reason why they should not accept part at least of the responsibility for any adverse effect which the drugs produced. It is easy to criticise the doctor who believes the claims made in the latest glossy circular or presented by a most personable representative. It is perhaps surprising how often doctors may accept advice on treatment from people who have never had the responsibility for the health of a single patient. On the other hand if pressed by his patient for "medicine" why blame the doctor for acceding to the importuning from someone who would not be sitting before him were he or she not the victim of some ill be it physical, mental or purely emotional. The actual nature of some drugs is probably of little importance compared with the confidence with which they are offered and the faith with which they are received. While cheap and inactive materials were distributed in this way relatively little harm was done to those who took the medicine or those who poured them away. Those who were really ill soon came back unrelieved and were sent on for a more careful scrutiny in hospital. Nowadays the wider distribution of powerful drugs with effects upon the sensorium may well cause untold harm not by their toxic side effects as much as by their pharmacological actions in masking symptoms. Indeed the doctor has been given powerful new weapons with which to modify bodily functions. While the main anxiety may at present be directed to the toxic side-effects which some drugs may produce in a few of those who receive them, the possible action of drugs in disguising signs and symptoms of serious disease seems to have excited little, if any, public discussion. It may be unwise to put powerful active drugs in the hands of those who cannot fully appreciate what such drugs could be capable of doing even in therapeutic doses. The world is becoming increasingly full of dangerous materials whether used in industry, on the roads, in the air, fields and homes. To reduce or prevent accidents the most essential step is the education of the user into the potentialities of what he is using. Drugs are no exception and the user here may be the doctor distributing them or the patient demanding or buying them.

Research into the Action of Drugs

Our idea of national priorities is indicated by the fact that the slice of the National Income put at the disposal of the University Grants

Committee does not equal that distributed as a subsidy to egg producers. Since it may be optimistic to expect too much from the better education of the prescribers and users of drugs it is necessary to see what can be done to ensure that new drugs are more effectively tested in order that those with undesirable toxic side-effects shall be excluded from general use. This may have the superficial attraction of diverting the responsibility for the safety of drugs from the user to the provider. In other words the manufacturers will be made responsible for the expensive work of testing their new materials more comprehensively for evidence of their possible harmful effects. However, no maunfacturer has the monopoly of wisdom in devising tests on animals that will provide evidence of the safety of his drug to man. He and his fellow manufacturers will therefore demand that those who wish to use the drugs or approve their distribution will tell them what tests and other research they should initiate in order to provide evidence of safety.

The first proposals are likely to be for the extended use of laboratory tests on animals. Many pharmacologists, both in industry and outside. are interested in the action of drugs and in studying the pharmacological activity of related compounds in order to provide evidence for or against current theories of modes of action. This will eventually provide a better understanding of physiological processes. Unfortunately few, if any, departments of pharmacology take any active interest in the study of the toxic side-effects of drugs. These, when they are detected, may result in a lessened interest in the study of a compound; indeed it is unlikely that a manufacturer would continue to produce such a material even for laboratory work. One result of this lack of interest in research in toxicology is that more and more reliance tends to be placed upon the performance of some patterns of tests on a few species of laboratory animals in order to see whether any unusual effect shows up. Certainly in the context of existing information there is sometimes little more that anyone can recommend as a method for examining a new compound for unsuspected side-effects. However unless more research into means of detecting toxic side-effects is undertaken this method of approach is likely to remain unimproved or even become less realistic. For example the recent outburst resulting mainly from the publication of "Silent Spring" has led The Scientific Advisory Committee to the President (1963) to recommend further laboratory tests of the toxicity towards mammals of new pesticides despite the fact that these are tested at least as thoroughly as many drugs. Furthermore people are exposed only to pesticides in minute fractions of the doses of which they receive drugs. It is recommended that tests on pesticides be continued for 2 generations of laboratory animals but there is no evidence whatever that these tests will reveal anything relevant to the possible hazards run by people exposed to such pesticides.

It is not difficult to make it appear that more is being done to make drugs safer by insisting that more animals are given doses for longer periods. All this work takes time, effort and expense and with the limited facilities available fewer can be spared for research. Since the

thalidomide disaster recommendations have been made for carrying out tests for a possible teratogenic effect on animals of new drugs (Somers, 1963). A great number of such tests have been, or are in the process of being, carried out. What is really needed, however, is an intense and diversified investigation of the biochemical and other mechanisms whereby thalidomide produces its teratogenic effects so that when more was known about this, some more rational tests might be devised to look for similar reactions from other compounds. Few will probably share the view that "teratogenicity—should not again be a danger to man; the test on rabbits should be applied as part of the routine pharmacological testing of all new drugs" (Macgregor and Perry, 1962). The danger will undoubtedly be least if the advice "to bar absolutely the use of new drugs by women who are believed to be in the early stages of pregnancy" (Woollam, 1962) is followed. An absolute faith in the predictive value of tests on animals will be misplaced; a failure to take some risks may not be the way to make progress.

Most toxic effects come as unpleasant surprises but each as it arises can offer an opportunity for research which may throw a great deal of light on the body processes in general. An excellent example of this was described in the last Conference Lecture when the story of the haemolysis produced in some patients receiving primaquine was described (Clark, 1962). This particular toxic side-effect has been thoroughly investigated and shown to be due to a heredity defect in the level of an enzyme in the red blood cells of certain members of the population (Beutler, 1960).

These studies explain the haemolytic reaction that may occur after exposure not only to the drug primaquine but also to a number of other drugs, substances used in industry or occurring naturally in plants (Larizza and others, 1960). Furthermore the observation that only a proportion of the exposed population is unduly sensitive to these substances is also explained. No progress whatever would have been made if the only result of the original observations had been that a dozen more tests should have been recommended to be carried out on laboratory animals in order to see whether a new drug produced haemolysis.

It is perhaps not unreasonable to believe that some of the other toxic side-effects of drugs that are seen only in man could be investigated more closely and yield as interesting a harvest of information as did the study of primaquine sensitivity. In the case of toxicity from drugs it seems that in almost all cases research will have to start with some study of man. Those substances with obvious side-effects in animals which are shown up in the preliminary toxicity tests will never be introduced. Thus it is to be hoped that committees set up to investigate the alleged incidence of toxic side-effects of drugs will also have the interest and be provided with the facilities to look into the mechanisms of toxic side-effects as well as just being responsible for recording their incidence and treating the victims.

It is not appropriate here to go into details as to how more research in toxicology should be promulgated. Each toxic substance is itself a

research tool and it may be particularly important to use a compound as a basis for such a piece of research if it seems to be a drug with great promise as a remedy yet apparently possessing some serious but limited side-effects. For example a candidate drug may produce an unusual incidence of one particular type of tumour in only one of several species of laboratory animal. It would be perfectly reasonable to reject such a material as a colour or flavouring agent in human food because its use would not benefit the consumer. However, if the use of such a drug held out hope of the relief of distressing symptoms or the eradication of a chronic infection for which no cure yet existed then it would be ridiculous to condemn it outright as a carcinogen unsuitable for use as a drug. Instead this would be an opportunity for intense research to understand why the sensitive species reacted in the way it did and on the basis of this to decide whether man might or might not be expected to react likewise.

The situation might be generally improved if the place of toxicology and the experimental toxicologist was reconsidered. At present there is a tendency for the man responsible for the toxicology to be at the end of the line saying "yes" and "no" to the onward passage of a new drug into the development of which his colleagues may have put much effort and on to which they and others have pinned great hopes. If research into toxicology was considered a vital part of research in pharmacology a much more productive arrangement might eventually emerge. If such a combined approach became common practice in departments of pharmacology as well as among those promoting new drugs then it would probably be needless and undesirable for Authority to insist that every new drug must be put through some programme of tests the significance of which in relation to the hazard to man was often very doubtful.

CONCLUSIONS

All human activity involves the incurring of some risks and the only ways in which these, if they are serious, should be reduced is by better education of those who run the risks or whose actions inflict such risks on others.

The further study of physiology, biochemistry and all the ancilliary medical sciences will eventually lead to better drugs and better means of using drugs. Prescribing doctors and patients have their share of responsibility in seeing that they find out about the drugs they give or those they request. Manufacturers and distributors have a big share of responsibility as soon as they make use of modern methods of sale persuasion. Every new drug that is made freely available for prescribers introduces an added risk of the production of side-effects. When these side-effects occur it may be argued that the proper reaction of Authority should be to make it more difficult to distribute so many different drugs, and that the most effective way will be by instituting time consuming and expensive toxicity testing and other scrutiny. The more widespread and large are the sales of a drug then so must the hazards from it increase.

A drug that has undoubted value for a relatively small number of people with a dangerous disorder may appear to be undesirable and dangerous for a significant number of those receiving it when it is prescribed to relieve a common condition of minor discomfort.

The Law has little or no place in protecting patients from dangerous drugs made available by *bona fide* manufacturers. It offers no more and no less of a safeguard against the wilful and criminal as it does in other walks of life.

Toxic hazards from chemicals used in industry, compounds used as pesticides, substances used as food additives and the toxic hazards from drugs have something in common but each have important distinctions. The toxicity of all the compounds used for these different purposes is often difficult to assess and to understand in strictly scientific terms. There is a serious danger that, by insisting on schemes of testing often prolonged and expensive but lacking a real scientific basis in their design and execution a better understanding of the toxic side-effects produced by drugs will never be obtained. While some general safeguards such as a minimum general testing of new drugs will long remain essential, this type of investigation should not be needlessly multiplied. Research in toxicology which is closely linked with pharmacology will be the only way in which safer drugs or drugs whose actions are more fully understood will eventually become available. The final word in any discussion on this subject can appropriately go to the Minister of Health (Mr. Enoch Powell) who in a recent debate included these words:

"I do not want to pass from this subject without saying emphatically that when we use the word 'safety' in this context we should not be understood to mean 'absolute safety'. Safety in this sphere is relative whatever may be the arrangements, whatever may be the law, it is relative to the illness and in the sense that there is no system that can be devised which will make doctors or scientists aware of what medicine and science have not yet suspected".

The Welfare State with all its benefits particularly in the field of the Health Service does possibly lead to a diminution of individual responsibility and a cry for protection against all possible risks or disturbances to comfort. The introduction and use of drugs involves many individual decisions. A form of blanket cover to guard against all risks will be either frustrating or deceptive. Increasing individual awareness is the only way of progress in this as in all fields of human activity.

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SCIENCE PAPERS

STERILISATION OF COLCHICINE INJECTION

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Colchicine solutions for injection can be sterilised by heating at 115° for 30 min., or by heating at 98 to 100° for 30 min. in the presence of phenylmercuric nitrate 0·002 per cent, or by filtration. Solutions in multi-dose containers may be prepared using phenol 0·5 per cent, phenylmercuric nitrate 0·001 per cent, or benzyl alcohol 1 per cent as bactericide. Sterilised solutions are stable for at least six months if protected from light.

Colchicine is used for the relief of pain in acute gout. It is usually administered in tablets but where a rapid response is required or where oral administration causes gastrointestinal disturbance, it may be given by intravenous injection, the usual dose being 2 to 4 ml. of a 0·1 per cent w/v solution in Water for Injection or Injection of Sodium Chloride. This investigation has been prompted by a number of enquiries to this department about methods for sterilising colchicine injection.

The structural formula of colchicine was proposed by Dewar (1945) and this was confirmed by King, de Vries and Pepinsky (1952) (I). Hydrolysis of the methoxyl substituent of ring C is readily brought about by dilute acids with the formation of the demethylated compound, colchiceine, which has been stated by Wallace (1961) to be ineffective in gout. More vigorous and prolonged hydrolysis by strong acids leads to the formation of trimethylcolchicinic acid and colchicinic acid. Trimethylcolchicinic acid has been shown by Wallace (1961) to be about as active as colchicine in the treatment of gout.

MeO, MeO, MeO, A, B, C, NHCOMe, O, OMe

(I)

On exposure to ultra-violet radiation, isomeric compounds known as lumicolchicines are formed, the formation of these isomers being accompanied by a change in the ultra-violet absorption spectrum (Grewe and Wulf, 1951).

Experimental and Results

Analytical Methods for Determining Decomposition of Colchicine

Analytical methods are required (i) to determine the amount of colchiceine formed by hydrolysis, and (ii) to detect the formation of lumicolchicines.

In this investigation, colchicine, B.P., was purified chromatographically, using a column of alumina saturated with benzene (Ashley and Harris, 1944). Colchiceine was prepared by the method of Cook and Loudon (1952). The purified colchicine recrystallised from ethyl acetate was in the form of pale yellow needles, m.p. 155°.

Determination of Colchiceine. The ultra-violet absorption spectra of colchicine and colchiceine are similar over the range 220 to 400 mμ (Bursian, 1938) and cannot therefore be used to distinguish colchiceine from colchicine.

A modification of the colorimetric method devised by Boyland and Mawson (1938) and later employed by Wood (1957) was found to be satisfactory. This is based upon the observation by Zeisel (1886) that colchiceine produces a green colour with ferric chloride solution whereas colchicine gives no colour reaction.

Extract a 0·1 per cent w/v solution of colchicine (10 ml.) with chloroform (3×3 ml. portions) and place the combined extracts in a 15 ml. graduated flask. Add dehydrated alcohol (4 ml.) and a 0·2 per cent w/v solution of ferric chloride (0·8 ml.) in chloroform, and immediately dilute to 15 ml. with chloroform. Allow to stand for exactly 5 min. and measure the light absorption at the maximum of about 460 mμ. To prepare the "solvent" blank solution, add dehydrated ethanol (4 ml.) to a 0·2 per cent w/v solution of ferric chloride (0·8 ml.) in chloroform, and dilute to 15 ml. with chloroform. Determine the proportion of colchiceine in the injection by reference to a calibration curve prepared using colchiceine solutions of known concentration.

It is necessary to allow the ferric chloride and the colchiceine to react for 5 min.; this is the minimum period necessary to produce a stable absorption maximum. Colchicine gave no reaction with the ferric chloride reagent.

The method was applied to six solutions containing 0·001 to 0·01 per cent w/v of colchiceine in water and agreement was obtained with the Beer-Lambert law. The method was then satisfactorily applied to a 0·1 per cent w/v solution of colchicine containing 0·001 to 0·01 per cent w/v of added colchiceine. Using the quantities given in the above method, it was found possible to detect 0·001 per cent w/v of colchiceine in a 0·1 per cent w/v solution of colchicine, this proportion of colchiceine representing 1 per cent decomposition of the colchicine.

Detection of lumicolchicines. A 0·1 per cent w/v aqueous solution of colchicine was exposed to ultra-violet radiation for 7 hr. and the ultra-violet absorption spectrum was observed before and after irradiation. A change was observed, the peak at 246 mμ shifting to 267 mμ, as reported by Grewe and Wulf (1951).

Sterilisation of Colchicine Solutions by Autoclaving

Preliminary work. Unless otherwise stated, solutions contained 0·1 per cent w/v of colchicine B.P. either in Water for Injection or in Injection of Sodium Chloride.

Solutions in both solvents were autoclaved at 115° in partly filled ampoules for from 30 min. up to 3 hr. No change in appearance could be detected except in solutions autoclaved for 3 hr.; in these solutions, a slight turbidity was observed but no colchiceine was detected. Solutions of two samples of colchicine from different commercial sources were prepared using the material (i) as received and (ii) after chromatographic purification; there was no difference in behaviour between different samples. Similar results were obtained at 121°.

Autoclaved solutions. Solutions in both solvents were filled into 2 ml. ampoules and autoclaved at 115° for 30 min. The clarity of solution, pH, colchiceine content and ultra-violet absorption spectrum were determined before and after autoclaving.

The pH of the solution in water rose from 5·9 to 6·5 and that in sodium chloride rose from 5·7 to 6·2 but there was no evidence of colchiceine formation or of changes in the ultra-violet absorption spectrum. The solutions remained clear and colourless.

Effects of pH. No measurable decomposition to colchiceine was observed in solutions of colchicine in both solvents adjusted to pH 4, 5, 6, 7, 8, 9, and 10 with 0·1N hydrochloric acid or 0·1N sodium hydroxide, and autoclaved at 115° for 30 min. In solutions at pH 3 decomposition was 2·4 per cent.

Sterilisation of Colchicine Solution by Heating with a Bactericide

Attempts were made to prepare a solution of colchicine in a 0·2 per cent w/v solution of chlorocresol and in a 0·002 per cent w/v solution of phenylmercuric nitrate. Colchicine dissolved relatively slowly in the chlorocresol solution to form a turbid solution; the turbidity remained on heating the solution to boiling.

Colchicine was compatible with the phenylmercuric nitrate. With solutions filled into 1 ml. ampoules and heated at 98 to 100° for 30 min., no turbidity or cloudiness, no change in pH or in the absorption spectrum, and no colchiceine could be detected.

Compatibility of Colchicine with Bactericides in Multi-dose Injections

To solutions in both solvents were added one of the following: phenol 0·5, cresol 0·3, chlorbutol 0·5, chlorocresol 0·1, phenylmercuric nitrate 0·001, and benzyl alcohol 1 per cent. The solutions were placed in Clinbritic bottles with rubber caps and autoclaved at 115° for 30 min. The results are in Table I. Incompatibilities occurred with cresol, chlorbutol and chlorocresol.

TABLE I

COMPATIBILITY OF COLCHICINE WITH BACTERICIDES IN MULTIDOSE INJECTIONS

Bactericide per cent concentration	Before autoclaving	After autoclaving
Phenol, 0·5	clear solution	clear solution
Cresol, 0·3	cloudy solution	cloudy solution
Chlorbutol, 0·5	clear solution	slightly cloudy solution
Chlorocresol, 0·1	cloudy solution	cloudy solution
Phenylmercuric nitrate, 0·001	clear solution	clear solution
Benzyl alcohol 1	clear solution	clear solution

Stability Tests on Colchicine Injection

Two samples of colchicine from different commercial sources were used to prepare colchicine injection; both samples complied with the B.P. specification: they were pale yellow amorphous powders having m.p. 153° and 153·5°.

Solutions. Four groups of colchicine injection were prepared using both solvents:

(*a*) Solutions in both solvents were filled into 2 ml. ampoules and sterilised by autoclaving at 115° for 30 min.

(*b*) Solutions were filled into 15 ml. rubber-capped Clinbritic bottles and sterilised by autoclaving at 115° for 30 min. The bactericides used were: phenol 0·5, phenylmercuric nitrate 0·001, and benzyl alcohol 1 per cent.

(*c*) Solutions containing 0·002 per cent w/v of phenylmercuric nitrate were filled into 2 ml. ampoules and sterilised by heating at 98 to 100° for 30 min.

(*d*) Solutions were sterilised by filtration through asbestos pads filled into 2 ml. ampoules.

Storage. The ampoules were stored for six months under the following conditions: in daylight, by a window; in the dark; in an incubator, at 25°, in the dark; in an incubator, at 37°, in the dark; and in a refrigerator, at 0 to 4°, in the dark.

Initially, after 1 week, 1 month, 3 months, and 6 months, the clarity of solution, the pH (determined electrometrically), the colchiceine content, and ultra-violet absorption spectrum were determined.

Results

Autoclaved solutions in ampoules. Solutions kept in the dark at ambient temperatures (at 7 to 27°) and at 0° to 4°, 25° and 37° remained clear and colourless; changes in pH were not significant; no change occurred in the ultra-violet absorption spectrum; there was no evidence of colchiceine formation.

Solutions kept in daylight showed no evidence of colchiceine formation but had become pale green after 3 months, with the formation of colourless crystals. These were identified as lumicolchicines (Grewe and Wulf, 1951). A significant fall in pH was observed. For example, the pH of one solution fell from 6·20 to 3·90 after 6 months; the pH of the solution prepared from the other sample of colchicine fell from 6·41 to 4·70 after 6 months. Autoclaved solutions in multidose containers, solutions heated with a bactericide, and unheated solutions sterilised by filtration gave similar results.

Examination of Colchicine Solutions Prepared Commercially

Four solutions in water were examined; two were autoclaved at 115° for 30 min., one was 7 years and the other 1 month old. One had been

heated at 98 to 100° for 30 min. and had been stored for 1 month. The fourth had been stored for 1 month in a 1 fl. oz. green ribbed bottle.

All solutions were clear and colourless. Their absorption spectrum corresponded to that of colchicine, there being no evidence of lumicolchicines. No colchiceine could be detected.

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SOME ANTIPYRETICS RELATED TO ASPIRIN AND PHENACETIN

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Aspirin and phenacetin are good analgesics and antipyretics, about whose defects, however, there is now considerable evidence. In small doses, both cause sensitisations, which may be trivial like skin rashes or fever, or severe like the blood dyscrasias or kidney damage. Also aspirin in large doses causes gastric bleeding. We have synthesised a number of substances related to aspirin and to phenacetin and have examined their pharmacology. In this communication we describe shortly their preparation, their acute toxicity and their antipyretic activity.

Benz-1,3-*oxazine*-2,4-*dione*. Carsalam was prepared by the method of Einhorn and Mettler (1902) with minor modifications. The product had m.p. 228° (lit., 227°).

m-*Ethoxyacetanilide*. The method of Reverdin and Lokietek (1915) was used substituting diethyl sulphate for ethyl bromide. The product had m.p. 97–98°.

m-*Hydroxyacetanilide* was prepared by the action of acetic anhydride on *m*-aminophenol (Reverdin and de Luc, 1914); m.p. 148–149°.

m-*Allyloxyacetanilide*. This was prepared essentially by the method of Arnold, McCool and Schultz (1942); m.p. 86–87° (lit., 87–88°).

m-*n-Pentyloxyacetanilide*. This was prepared in a similar fashion to the above by the action of n-pentyl bromide on *N*-acetyl-*m*-aminophenol. The material crystallised in colourless needles from light petroleum (b.p. 60–80°); m.p. 77°. Found: C, 70·1; H, 8·8; N, 6·0 per cent. $C_{12}H_{19}O_2N$ requires C, 70·6; H, 8·7; N, 6·3 per cent.

Acute Toxicity

Carsalam in mice. Groups of 5 fasted mice were given a single oral dose of 0·67 to 3·3 g./kg. suspended in 5 per cent acacia mucilage and the number of deaths within 5 days was recorded.

Carsalam in rats. Groups of 10 male rats were treated orally with 0·35 to 1·0 g./kg. in 5 per cent acacia mucilage (2 ml./100 g.) and the number of deaths within 5 days was recorded. All results were calculated by the method of Karber (1931) and Miller and Tainter (1944).

m-*Ethoxyacetanilide* was administered orally in a 5 per cent acacia mucilage to groups of not less than 10 mice.

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Antipyretic Action

Carsalam, aspirin, chlorthenoxazine and salicylamide were compared by a modification of the method of Brownlee (1937). The drugs were given orally and the pyrogens injected immediately afterwards instead of giving the antipyretics 2 hr. after the pyrogens.

Groups of 3 rabbits were given by stomach tube, 500 mg./kg. of one or other of the drugs in suspension in 5 ml./kg. of 2 per cent acacia mucilage containing 0·1 per cent of polysorbate 80. Other groups were given the mucilage as controls. Immediately afterwards each animal was injected into an ear vein with 1·0 ml./kg. of TAB vaccine (protein shock, 500 million organisms per ml.). The rabbits had been deprived of food but not water overnight and both were withheld during tests. Temperatures were recorded with a thermistor apparatus (Whittet, 1958) every 15 min. for at least 1 hr. before and 3 hr. after treatment. The tests were repeated as cross-overs until each of 12 rabbits had received each drug and the control. The temperatures are plotted as a comparison with the controls as a straight line at 0° (Brownlee, 1937) in Fig. 1. From the curves the reduction in temperature can be calculated graphically in a manner similar to that used for fever indices (Beeson, 1947). We have called these "antipyretic indices"; they are shown in Table I.

TABLE I

ANTIPYRETIC INDICES (3 HR.) OF CARSALAM, PHENACETIN AND RELATED DRUGS

Drug	Antipyretic index
Carsalam	270 (12)
Aspirin	143 (12)
Salicylamide	35 (12)
Chlorthenoxazine	−1 (12)
Phenacetin	70 (12)
Paracetamol	150 (12)
m-Ethoxyacetanilide	152 (36)
m-Hydroxyacetanilide	120 (12)
m-Allyloxyacetanilide	108 (24)
m-n-Pentyloxyacetanilide	64 (24)

Figures in parenthesis are number of tests.
The antipyretic index is the amount of reduction of fever, compared with controls, caused by a drug in a definite period after treatment with the antipyretic and injection with pyrogens (3 hr. in the above cases.) It is measured by plotting the control as a straight line at 0° and the temperatures of animals given antipyretics as differences from this. The area between the control and temperature-reduction line gives a measure of the antipyretic effect.

Further groups of 3 rabbits were given, by the same technique, doses of 50, 100, 200 and 400 mg./kg. of carsalam, of aspirin and of 5 ml./kg. of mucilage as controls. Each was injected with 0·1ml./kg. of TAB vaccine. The antipyretic indices were again determined graphically (Table II).

Carsalam had an appreciable hypothermic effect and this action was therefore compared with that of aspirin. Groups of 3 rabbits were given 50, 100, 200 and 400 mg./kg. of carsalam and 50 and 400 mg./kg. of aspirin and their temperatures were recorded for at least 1 hr. before and 4½ hr. after treatment. The tests were repeated as cross-overs. The mean temperature changes were plotted against time for 4½ hr. after treatment and the amount of hypothermia produced by each dose during that period was measured graphically. We have called this the "hypothermic index". (Table II).

TABLE II

ANTIPYRETIC AND HYPOTHERMIC INDICES FOR VARIOUS DOSES OF CARSALAM AND ASPIRIN

Dose mg./kg.	Antipyretic index*		Hypothermic index†	
	Carsalam	Aspirin	Carsalam	Aspirin
50	− 48 (12)	23 (12)	37 (12)	− 23 (6)
100	− 10 (12)	− 18 (12)	62 (12)	—
200	37 (12)	82 (12)	161 (12)	—
400	172 (12)	145 (12)	201 (12)	13 (6)

Figures in parenthesis are number of tests.
* Three hr.
† The hypothermic index is the amount of reduction in temperature as compared with the initial temperature, in a definite period after treatment with the drug (4½ hr. in the above instances). It is determined by measuring the area between the mean initial temperature drawn as a straight line and the curves showing changes in temperature.

Tests were also carried out by the original Brownlee method using doses of 500 mg./kg. of carsalam and 0·1 ml./kg. of vaccine.

m-*Hydroxyacetanilide derivatives.* The antipyretic activities of these compounds were compared with those of phenacetin and paracetamol by the modified Brownlee method. The temperature changes are plotted as a comparison with the controls as a straight line at 0° in Fig. 2. Their antipyretic indices are shown in Table I. Tests were also made on *m*-ethoxyacetanilide by the original Brownlee method using 500 mg./kg. of the compound and 0·1 ml./kg. of TAB vaccine.

RESULTS AND DISCUSSION

Carsalam

Acute toxicity. The oral toxicity to mice was between 1·0 and 1·5 g./kg. This is similar to that of aspirin (Spector, 1956, gives 1·1 g./kg.) but that found for rats, 750 mg., was much greater than that of aspirin (Spector gives 1·36 g./kg.). This significant species difference is not unusual but the compound needs to be tested further on different species.

Antipyretic action. Carsalam has approximately 1·7 times the antipyretic effect of aspirin in a dose of 500 mg./kg. (Fig. 1). Salicylamide and chlorthenoxazine had little antipyretic action in this dose.

Hypothermic action. Carsalam 50 mg./kg., produced a reduction of temperature of approximately 0·6° for about 1½ hr. after oral administration and with a dose of 400 mg./kg. the temperature was nearly 2° below its initial value after 4½ hr. The original Brownlee method showed that carsalam in doses of 500 mg./kg. rapidly reduced fever already present. Rabbits given doses of 400 to 500 mg./kg. of carsalam showed marked diuresis and frequently became limp for a few hours.

m-*Hydroxyacetanilide Derivatives*

Acute toxicity of m-*ethoxyacetanilide.* Doses of 200 and 500 mg./kg. of this compound caused no deaths and a further dose of 1 g./kg. caused only 1 death in a group of 20 mice. The oral LD50 for mice is, therefore, clearly considerably greater than 1 g./kg.

The antipyretic indices obtained from Fig. 2 show that *m*-ethoxyacetanilide has an effect about twice that of phenacetin and similar to

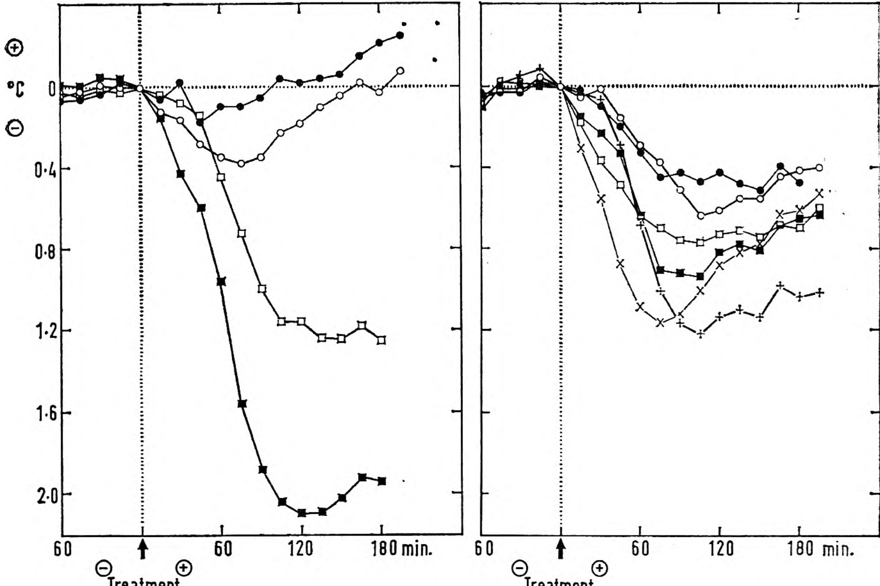

FIG. 1. Antipyretic action of carsalam, aspirin, chlorthenoxazine and salicylamide Control at 0° C. ■—■ = Carsalam. □—□ = Aspirin. ●—● = Chlorthenoxazine. ○—○ = Salicylamide. Each point is the mean of 12 tests.

FIG. 2. Antipyretic action of phenacetin, paracetamol and compounds 747, 749, 875 and 880. Controls at 0° C. 48 tests. ○—○ = Phenacetin 12 tests. +—+ = Paracetamol 12 tests. ×—× = *m*-ethoxyacetanilide 36 tests. ■—■ = *m*-hydroxyacetanilide 12 tests. □—□ = *m*-allyloxyacetanilide 24 tests. ●—● = *m*-pentyloxyacetanilide. 24 tests.

that of paracetamol. The effect of the allyloxy derivative is approximately 50 per cent greater than that of phenacetin, whilst the n-pentyloxy compound is slightly less effective. By the original Brownlee method *m*-ethoxyacetanilide 500 mg./kg., rapidly reduced fever already present.

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AN ACCELERATED STORAGE TEST WITH PROGRAMMED TEMPERATURE RISE

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The rate constant of a reaction at room temperature and the activation energy can be calculated from values of the concentration of the reactant as a function of time. Measurements are made while the temperature of the reacting system is raised in accordance with a predetermined programme. The method makes use of the Arrhenius equation, but it does not suffer from the disadvantages of the usual method in which several experiments are done at fixed elevated temperatures.

THE stability of a preparation at room temperature can be predicted by measuring the rate of decomposition at two or more higher temperatures, and the method has found important applications in pharmacy (Garrett, 1962).

It is assumed that the relation between rate constant k and temperature T (° K.) can be described by the Arrhenius equation,

$$\frac{d \log k}{d\,(1/T)} = -\frac{E}{2{\cdot}303\,R}$$

where E is the activation energy for the reaction, R is the molar gas constant, and logarithms are taken to the base 10. The activation energy can be calculated if the rate constant is known at two temperatures; alternatively, from a knowledge of the rate constant at one temperature and the activation energy, the rate constant at some other temperature can be calculated. This is conveniently done by a graphical plot of log k as a function of 1/T, although Tootill (1961) has pointed out the advantages of a statistical approach.

If moderately high temperatures, such as 60° or 80°, are chosen for the experiment, the rate of reaction may be increased by a factor of ten or more over that at room temperature, and so the "shelf life" at room temperature, which may be years for some pharmaceutical preparations, can be predicted from the results of a few weeks' work. The method suffers from the disadvantage that the rate of decomposition should be known at least approximately in advance, so that suitable elevated temperatures can be chosen, and there is a risk that at the higher temperatures used, the reaction mechanism may alter. A method is now proposed that largely avoids these difficulties.

A single experiment is done, replicated if thought desirable, in which the temperature of the preparation is steadily raised in accordance with a predetermined programme, and samples are withdrawn at intervals and analysed in the usual way. This provides the information necessary to calculate the activation energy of the reaction and the rate constant at any temperature.

Let t represent the time and c the concentration of the reactant; T_0 and T_t are the temperatures (° K.) at the start and at time t, and k_0 and k_t are the corresponding rate constants. The rise of temperature is programmed so that the reciprocal of the temperature varies logarithmically with time

$$1/T_0 - 1/T_t = 2{\cdot}303 \text{ b} \log (1 + t)$$

where b is a constant of proportionality which can be chosen as desired.

Since $d(\log k)/d(1/T) = -E/2{\cdot}303R$, then

$$\begin{aligned} \log k_t &= \log k_0 + (E/2{\cdot}303R)(1/T_0 - 1/T_t) \\ &= \log k_0 + (Eb/R) \log (1 + t) \\ \therefore k_t &= k_0.(1 + t)^{Eb/R} \end{aligned}$$

If first-order kinetics are assumed, $-dc/dt = k.c$, and

$$-\int_{c_0}^{c_t} \frac{dc}{c} = \int_0^t k.dt$$

$$\begin{aligned} \therefore 2{\cdot}303 \log (c_0/c_t) &= k_0 \int_0^t (1 + t)^{Eb/R}.dt \\ &= \frac{k_0.[(1 + t)^{1 + Eb/R} - 1]}{1 + Eb/R} \\ &= \frac{k_0.(1 + t)^{1 + Eb/R}.[1 - (k_0/k_t)^{1 + R/Eb}]}{1 + Eb/R} \end{aligned}$$

$$\begin{aligned} \therefore \log [2{\cdot}303 \log (c_0/c_t)] = \log k_0 &- \log (1 + Eb/R) \\ &+ (1 + Eb/R) \log (1 + t) \\ &+ \log [1 - (k_0/k_t)^{1 + R/Eb}] \end{aligned}$$

If other orders of reaction are assumed, similar equations are obtained. In general,

$$\log f = \log k_0 - \log (1 + Eb/R) + (1 + Eb/R) \log (1 + t) + \log [1 - (k_0/k_t)^{1 + R/Eb}]$$

where f is $(c_0 - c_t)$ for zero order, $[2{\cdot}303 \log (c_0/c_t)]$ for first order, $(1/c_t - 1/c_0)$ for second order, etc.

The final term on the right-hand side of the equation varies with time. Just after the start of the experiment, it is large and negative, but it rapidly tends to zero as the reaction proceeds, and it becomes negligible as k_t becomes substantially greater than k_0.

A graph of log f as a function of log (1 + t) will therefore be a straight line from that time after which k_0 is negligible in comparison with k_t. The slope of the straight line is equal to (1 + Eb/R). Since b, the programme constant, and R are known, the activation energy E can be calculated. If the graph is not a straight line after the initial period during which k_t is only slightly greater than k_0, this may be because the

wrong order of reaction has been assumed, or because the mechanism changes as the temperature is raised. The intercept at $\log (1 + t) = 0$ is equal to $\log k_0 - \log (1 + Eb/R)$, and so if the slope and the intercept are measured, the rate constant k_0 at temperature T_0 can be calculated. From k_0 and E, the rate constant at any other temperature can be calculated.

It is thus possible from a single experiment to estimate the activation energy for the reaction and the rate constant at any temperature. The straight-line plot provides a built-in check that the correct order of reaction has been assumed, and that this and the activation energy are independent of temperature over the range studied. If the log f *vs.* log $(1 + t)$ graph is initially straight and then curls over, this may indicate a change of mechanism at the higher temperature, and the slope and intercept of the line through the points at the lower temperatures should be used for the calculations.

It is not necessary for approximate values of k_0 and E to be known in advance. The starting and stopping temperatures of the programme can be chosen according to experimental convenience, and the programme constant b can be selected according to the time available for the experiment.

If desired, the experiment can be stopped as soon as the requisite number of points have been plotted to enable the slope and the intercept to be measured with sufficient accuracy, and so high temperatures with their attendant difficulties may be avoided.

Experimental

The method has been tested by experiments on the first-order decomposition of riboflavine and of sucrose in aqueous solution.

Riboflavine. A 10^{-4}M solution of riboflavine in 0·05N sodium hydroxide was used. A value of $0{\cdot}0005/2{\cdot}303 = 2{\cdot}171 \times 10^{-4}$/deg. was selected for b, with $1/T_0 = 0{\cdot}00350$/deg., so that the temperature was programmed to rise from 12·5° to 55° in 7 hr. (Fig. 1). The riboflavine solution was distributed in 10 ml. portions into 25 ml. flasks in a water-bath, and the temperature was raised during the day by manual adjustment of the bath thermostat. Initially, the temperature rise was about 0·25° per min., and adjustment was needed every 1 or 2 min.; later, the rate fell to about 0·05° per min., and adjustment was made only every 5 or 10 min. A thermometer in one of the flasks was used to monitor the temperature.

For analysis, the method of Guttman (1962) was used. A 10 ml. aliquot was cooled under the tap and diluted to 25 ml. with N acetic acid, and the extinction was measured at 445 mμ in 1 cm. cells in a Hilger and Watts "Uvispek" spectrophotometer. The concentration c is directly proportional to the extinction or absorbance A. The experiment, including the analyses, was done in subdued light.

The graph of $\log [2{\cdot}303 \log (A_0/A_t)] = \log [2{\cdot}303 \log (c_0/c_t)]$ as a function of $\log (1 + t)$ is shown in Fig. 2. The times of removal of samples for analysis are spaced logarithmically, so that the points are spread uniformly across the graph. The slope is 2·95 and the intercept at log

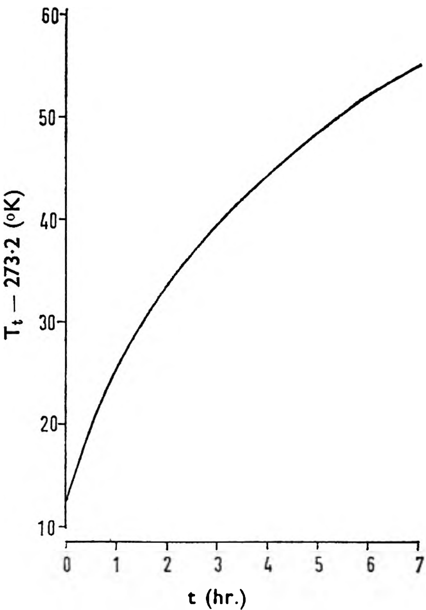

FIG. 1. Temperature rise according to the programme $(0{\cdot}00350 - 1/T_t) = 0{\cdot}0005 \log (1 + t)$, with T_t in °K, and t in hr.

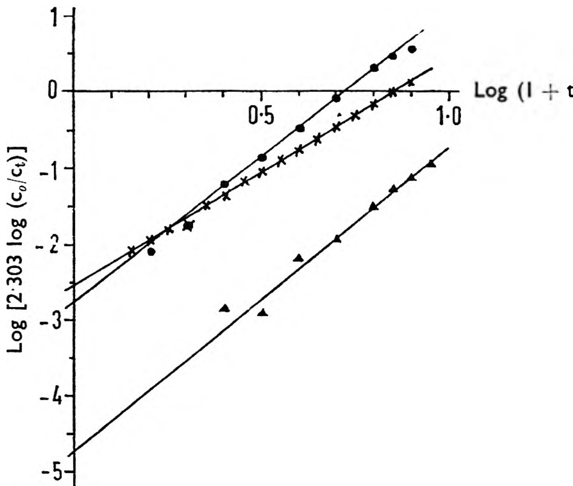

FIG. 2. Decomposition during temperature rise according to the programme $(0{\cdot}00350 - 1/T_t) = 0{\cdot}0005 \log (1 + t)$, with T_t in °K and t in hr.

× —— × riboflavine in N/20 sodium hydroxide.
● —— ● sucrose in N/6 hydrochloric acid.
▲ —— ▲ sucrose in N/600 hydrochloric acid.

$(1 + t) = 0$ is $-2{\cdot}55$. Therefore the activation energy $E = 1{\cdot}987 \times 10^{-3} \times (2{\cdot}95 - 1)/(2{\cdot}171 \times 10^{-4}) = 17{\cdot}85$ kcal./mole; $\log k_0 = -2{\cdot}55 + \log 2{\cdot}95 = -2{\cdot}08$ and so $k_0 = 0{\cdot}0083$/hr. The rate constant at 20° calculated from E and k_0 is 0·018/hr. The rate constant was also measured directly in a separate experiment by determination of d(log c)/dt at a constant temperature of 20° and was found to be 0·019/hr. A value of 0·016/hr. at 20° can be interpolated from the results for the decomposition of similar riboflavine solutions reported by Guttman (1962); his value for the activation energy was about 19·2 kcal./mole.

Sucrose (i). A 40 per cent w/v solution of sucrose in 0·0002 per cent w/v phenylmercuric nitrate solution was prepared and 20 ml. portions were distributed into flasks. To each was added 10 ml. of 0·5N hydrochloric acid, and the programmed temperature rise was started, with $1/T_0 = 0{\cdot}00350$/deg. and $b = 2{\cdot}171 \times 10^{-4}$/deg.

For analysis, a flask was removed from the bath and cooled under the tap, and the solution was diluted to 50 ml. with 0·26N sodium hydroxide. The optical rotation α was measured at 546·1 mμ in a 20 cm. tube in a Hilger and Watts photoelectric polarimeter. The concentration c_t is directly proportional to $(\alpha_t - \alpha_\infty)$, where α_∞ is the rotation when hydrolysis is complete and is approximately $-0{\cdot}32\,\alpha_0$, and so $\log\left[2{\cdot}303 \log \frac{\alpha_0 - \alpha_\infty}{\alpha_t - \alpha_\infty}\right]$ $= \log [2{\cdot}303 \log (c_0/c_t)]$ was plotted as a function of $\log (1 + t)$.

The slope of the graph (see Fig. 2) is 3·82 and the intercept at $\log (1 + t) = 0$ is $-2{\cdot}75$. Therefore the activation energy is 25·8 kcal./mole, and the rate constant at 20° is predicted to be 0·021/hr. The rate constant at 20° measured directly was found to be 0·024/hr. A value of 0·020/hr. can be interpolated from the results for the decomposition of similar alkaline sucrose solutions reported by Jackson and Gillis (1920); their value for the activation energy was about 25·5 kcal./mole.

Sucrose (ii). Another sucrose decomposition experiment was done, but with a concentration of hydrochloric acid only about one-hundredth of that in experiment (*i*). The slope of the graph (see Fig. 2) is 4·02 and the intercept at $\log (1 + t) = 0$ is $-4{\cdot}74$. Therefore the activation energy is 27·6 kcal./mole, and the rate constant at 20° is predicted to be 0·00022/hr. The rate constant at 20° measured directly was found to be 0·00023/hr.

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The paper was presented by the AUTHOR.

THE INSTRUMENTATION OF A ROTARY TABLET MACHINE

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A rotary tablet machine has been instrumented to measure the forces operating during the compression cycle. A radio link was used to transmit to a recording unit the effect of pressure on strain gauges attached to the punches. Sodium chloride and aspirin were compressed at various pressures and the results compared, using strength and porosity data, with tablets of the same materials prepared on an eccentric machine.

The use of small-resistance strain gauges bonded to the punches or the frame of a single punch, eccentric tablet machine has allowed accurate measurement of the forces exerted by the upper punch and transmitted to the lower punch (Higuchi, Nelson and Busse, 1954; Hasegawa, 1959; Shotton and Ganderton, 1960; Riad and Zobel, 1962; Fuhrer, 1962). Such instrumentation has led to some success in the correlation of the forces of compression with various tablet properties, such as strength, disintegration, porosity, static electrification and weight variation. Since the eccentric machine has been almost completely replaced by the rotary tablet machine, it seemed desirable to compare the two types of machine and the effect of pressure being applied by the movement of both punches. Seth and Munzel (1958) attempted a comparison of tablets prepared on eccentric and rotary machines by compressing different portions of the same granulation to the same density on each machine. However, as the compressional forces were not determined, the comparison was incomplete.

This paper describes a rotary tablet machine instrumented to give simultaneous records of the pressure exerted by the upper and lower punches. Two crystalline materials, sodium chloride and aspirin, were compressed, the compression cycle was analysed, and the results compared, using crushing strength and porosity data, with compression on an eccentric machine.

The Instrumentation of a Rotary Tablet Machine

A 16-punch, rotary tablet machine (Manesty Machines Ltd. Type D.3) blanked off to leave one punch and die set was kindly made available by Burroughs Wellcome and Co. Ltd. of Dartford.

In an eccentric machine, strain gauges were mounted on the punches and the signals obtained by direct connection, but the movement of the turret of a rotary machine precludes the use of fixed leads. This problem offered two practicable alternatives: either the use of slip rings or radio telemetry. A slip ring system required such modification of the machine as to confine instrumentation to one particular machine. Also, electrical noise resulting from loose powder adhering to the slip rings could only

be avoided by careful screening or the use of an enclosed ring, such as the mercury contact slip ring. For these reasons, the slip ring system was rejected in favour of radio telemetry.

Two strain gauges (H. Tinsley Co. Ltd. Type 6J. Gauge factor 2·19. Resistance 100 ohms) were bonded to the shank of a plane faced, half-inch punch and connected in series. A small terminal block was cemented between the gauges with "Araldite" and leads were attached. The strain gauges formed the active arm of a Wheatstone bridge. The other three arms, consisting of two matching strain gauges as a reference arm and two high stability 200 ohm carbon resistors, were mounted on a small plastic plate with an oscillator, amplifier and driving batteries. A variable resistance was fixed in parallel to the reference arm for balancing the bridge. The bridge was activated by an alternating current at approximately 7 V and about 550 c./sec. supplied by a small solid state oscillator (Telephone Manufacturing Co. Ltd. Type SO11). The bridge output was amplified (Telephone Manufacturing Co. Ltd. Solid state amplifier. Type SA 10) and the signal passed to the transmitter through the condensers C1 and C2 (Fig. 2).

The plate was fitted beneath the upper punch casting, with the amplifier and oscillator inserted into vacant punch holes, and held in position by screws, which passed through the casting and held a second plate in position on the top. This plate carried a calibrating resistance, which when connected across the active arm of the bridge by its associated switch, gave an out of balance of 0·15 per cent.

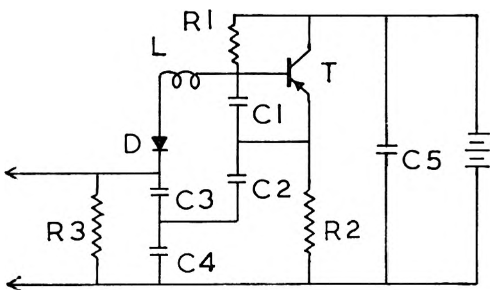

FIG. 1. Modified Gouriet Oscillator. R_1 50 kΩ. R_2 1 kΩ. R_3 100 kΩ. C1, C2 and C4 30 Pf. C3 and C5 1000 Pf. T Transistor OC171 (Mullard). D Diode OAZD (Mullard). L Coil. Two turns 0·5 in. diameter. 20 S.W.G. copper wire.

The transmitter circuit was similar to that described by Wolff, McCall and Baker (1962). The circuit, shown in Fig. 1, was modified for use with a standard domestic tuning unit, operating in the region of 100 Mc./sec. The transmitter was encapsulated in "Araldite" and this and its driving batteries were housed in adjacent punch holes. In this way a compact system was constructed which cleared the stationary feed frame. A block diagram of the transmitting apparatus is given in Fig. 2.

The receiving apparatus consisted of a tuning unit (H. J. Leak Co. Ltd. Type Troughline II), the output of which was modified by a full wave rectifier bridge. The output was fed into a recorder (New Electronic

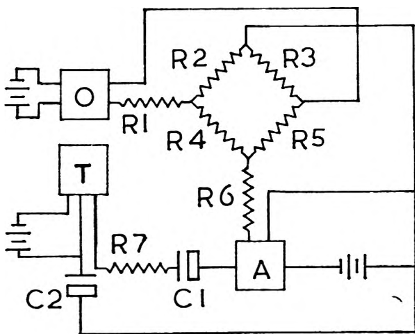

FIG. 2. Block diagram of complete transmitting apparatus. R1, R2, R3, R4 and R5 200 Ω. R6 1 kΩ. R7 2 kΩ. C1 and C2 4 μF (electrolytic). O Oscillator. T Transmitter. A Amplifier. Voltages. O 8 V. T 6·5 V. A 8 V.

Products. Ultra-violet Recorder Type 1050) using resistors to comply with the impedance of the components. The galvanometers used with the recorder had a natural frequency of 100 c./sec. (Southern Measuring Instruments. Type SMI V). A block diagram of the receiving circuit is given in Fig. 3.

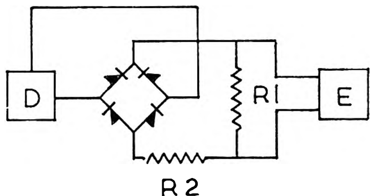

FIG. 3. Block diagram of receiving apparatus. D Tuner. E Recorder. R1 250 Ω. R2 10 kΩ. Bridge 4 × OA10 Diode. (Mullard.)

A similar bottom punch system, differing only in the spatial layout of the components, was constructed and fitted beneath the die-plate of the tablet machine. The transmitter occupied a vacant die space. Since the passage of the metal feed frame over the transmitter severely modulated the frequency, the transmitter was so placed to be clear of the feed frame during the compression cycle.

A dipole aerial, shaped to follow the circumference of the die-plate and having an uninterrupted view of the radiating coils of both transmitters was fixed to the machine.

In order to record simultaneously the upper and lower punch forces, the components of the transmitters were so chosen to give a basic frequency of 104 Mc./sec. for the upper punch transmitter and 96 Mc./sec. for the lower punch transmitter. Thus, by suitable tuning of the receiving units, complete separation of the two signals was obtained.

The punches were calibrated separately by mounting them on a standardised strain column and compressing the assembly between the platens of a small hydraulic press. After the construction of force-deflexion series from 0–3,000 kg., the load was released and an out-of-balance of 0·15 per cent was introduced into the punch bridge circuit by switching a

suitable resistance into parallel with the active arm of the bridge. The associated deflexion was recorded and evaluated in terms of force. Thus, the deflexions recorded during the compression of a tablet could be evaluated by comparison with the switched out-of-balance deflexion.

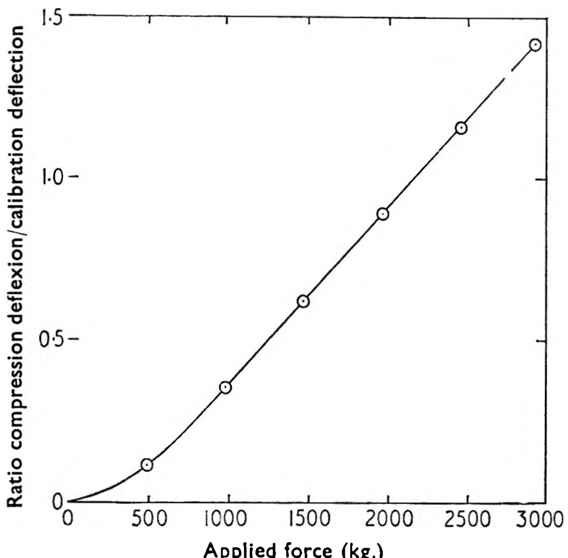

FIG. 4. The upper punch calibration curve.

The relation between galvanometer deflexion and applied load is given in Fig. 4. This relation is linear over the force range 800–3,000 kg. but shows curvature to the origin below 800 kg. The experimental work was confined to this range with two exceptions when forces just below 800 kg. were recorded. The calibration deflexion was reproducible and gave values of 2,080 ± 60 kg. for the upper punch and 1,930 ± 30 kg. for the lower punch in eight independent trials.

Aspirin and sodium chloride were selected for the initial study because their compression properties have been extensively studied with an eccentric machine. Aspirin is easily compressed to a low porosity, whereas sodium chloride shows considerable resistance to consolidation. When compressed on an eccentric machine, both materials give high die wall friction effects resulting in marked differences between the upper and lower punch forces. Both, however, can be satisfactorily compressed without the aid of lubricants.

EXPERIMENTAL

Sodium chloride and aspirin were sieved and 30–40 mesh fractions of each material were selected. For most of the work, the feed frame was not used. Weighed samples of the material were fed into the die by hand with the die-place diametrically opposite the compression point. This procedure was adopted so that the recording system could be calibrated immediately before or after compression.

The weight of the samples was chosen to give a tablet 0·4 cm. thick at zero porosity. Six or eight tablets were prepared at each pressure level and the pressure range 600–2,000 kg./cm.^{2} was investigated at seven or eight points. To reduce systematic errors, intermediate pressures were first investigated. Compression at high pressures was followed by a low pressure study, with the production of tablets at the original machine setting as a check. The tablets were collected and stored in closed containers for 6 hr. They were then weighed, the thickness and diameter measured, and the strength estimated using a crushing test previously described by Shotton and Ganderton (1960). The duration of the compression cycle was evaluated from the galvanometer records.

The feed frame was fitted to the machine for the production of aspirin tablets over the same pressure range. The weight, thickness, strength and duration of compression cycle were evaluated as before.

Finally, for comparison with the tablets produced on the rotary tablet machine, the same samples of aspirin and sodium chloride were compressed on an eccentric machine using direct leads.

As the projection of the linear portion of the graph showing the relation between applied load and galvanometer deflexion did not pass through the origin a graphical correction was made for computing the load.

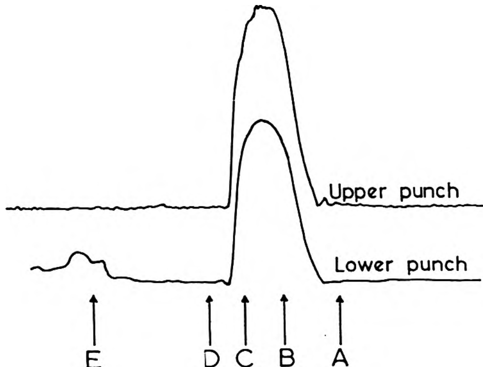

FIG. 5. Record of the compression of a tablet of sodium chloride. See text for explanation of symbols.

Details of Compression Cycle

Fig. 5 is an example of the traces obtained from the upper and lower punch of the rotary tablet machine during the compression cycle. The upper punch after moving down its guide, rests on the powder in the die, meets the pressure-wheel at position A and is forced downwards under the pressure wheel, to position B when the leading edge of the flat punch top reaches bottom dead centre. The flat punch top passes under bottom dead centre and position C represents the point at which the flat punch top leaves bottom dead centre. At position D, the punch leaves the pressure-wheel and position E represents the ejection of the tablet. These approximate punch positions were obtained visually by rotating the machine by hand.

The force exerted by the pressure wheel at positions B and C is less than that exerted when the centre of the punch passes bottom dead centre as the force at B and C will be exerted at an angle to the vertical and only the vertical component will be measured at the punch tip.

Results

From the paper speed of the recorder the approximate duration of the compression cycle was found to be 0·16 sec. for all tablets produced on the rotary tablet machine. For the eccentric machine, the duration was about 0·14 sec. for aspirin and about 0·11 sec. for sodium chloride, these values being variable due to turning the machine by hand.

The results for sodium chloride compressed on both rotary and eccentric machines are summarised in Table I. The results for aspirin are given in

TABLE I

THE COMPRESSION OF SODIUM CHLORIDE

Upper punch pressure Pa, kg./cm.^{2}	Lower punch pressure Pb, kg./cm.^{2}	Mean compaction pressure Pm, kg./cm.^{2}	$\frac{Pb}{Pa}$	Weight of tablet, g.	Porosity, per cent	Crushing strength Fc, kg.
		Rotary tablet machine				
2,190	1,980	2,090	0·90	1·0957	4·7	22·9
1,990	1,810	1,900	0·91	1·0970	5·5	23·7
1,740	1,560	1,650	0·90	1·0961	7·0	18·7
1,550	1,400	1,480	0·90	1·0960	8·0	15·8
1,370	1,230	1,300	0·90	1·0956	9·7	15·0
1,070	1,000	1,040	0·94	1·0933	13·1	11·1
820	760	790	0·92	1·0940	17·2	7·5
630	550	590	0·88	1·0919	21·2	5·5
		Eccentric machine				
1,970	1,390	1,680	0·70	1·0884	6·1	13·7
1,570	1,170	1,370	0·74	1·0921	8·3	12·9
1,300	960	1,130	0·74	1·0913	10·8	10·2
990	760	880	0·77	1·0942	14·0	6·8
550	430	490	0·78	1·0902	21·5	3·7

All values are the mean of six results

TABLE II

THE COMPRESSION OF ASPIRIN

Upper punch pressure Pa, kg./cm.^{2}	Lower punch pressure Pb, kg./cm.^{2}	Mean compaction pressure, kg./cm.^{2}	$\frac{Pb}{Pa}$	Weight of tablet, g.	Porosity p, per cent	Crushing strength Fc, kg.
		Rotary tablet machine				
2,080	1,860	1,970	0·89	0·7182	4·7	5·7
1,720	1,510	1,620	0·88	0·7191	5·3	5·6
1,520	1,340	1,430	0·89	0·7181	5·5	5·0
1,350	1,190	1,270	0·88	0·7172	5·9	4·6
1,080	950	1,020	0·88	0·7114	6·8	3·8
820	740	780	0·89	0·7117	7·4	3·3
650	580	620	0·89	0·7166	7·6	2·7
		Eccentric tablet machine				
2,370	1,780	2,080	0·75	0·7202	4·7	6·1
2,140	1,560	1,850	0·73	0·7211	4·7	5·9
1,860	1,310	1,590	0·71	0·7212	4·9	5·3
1,330	920	1,120	0·69	0·7210	5·4	4·5
820	550	690	0·67	0·7185	6·5	3·6

All values are the mean of six results

Table II and the results obtained by frame feeding aspirin to a rotary machine are given in Table III.

TABLE III

THE COMPRESSION OF ASPIRIN ON A ROTARY TABLET MACHINE USING A FEED FRAME

Upper punch pressure Pa, kg./cm.²	Lower punch pressure Pb, kg./cm.²	Mean compaction pressure Pm, kg./cm.²	Pb/Pa	Weight of tablet, g.	Porosity p, per cent	Crushing strength Fc, kg.
2,160	1,920	2,040	0·89	0·7165	5·5	5·1
1,740	1,380	1,560	0·79	0·7138	5·9	4·5
1,400	1,160	1,280	0·83	0·7117	6·3	3·6
960	820	890	0·85	0·7223	5·8	3·1
810	730	770	0·90	0·7051	7·0	2·6
680	590	640	0·87	0·7242	7·7	2·3

All values are the mean of 10 results

The results obtained from hand feeding and compressing aspirin and sodium chloride on the two machines were compared using the relation between porosity and mean compaction pressure (Fig. 6) and between crushing strength and mean compaction pressure (Fig. 7).

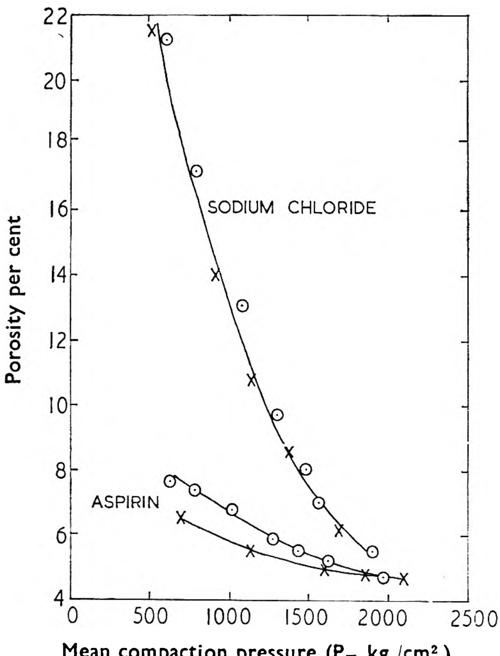

FIG. 6. Relation between porosity and mean compaction pressure for tablets of aspirin and sodium chloride. × Results from the eccentric machine, ○ Results from the rotary machine.

DISCUSSION

The results given in Tables I and II indicate that the ratio between the lower punch pressure and the upper punch pressure, P_b/P_a, during compression on a rotary machine was greater than during compression on an

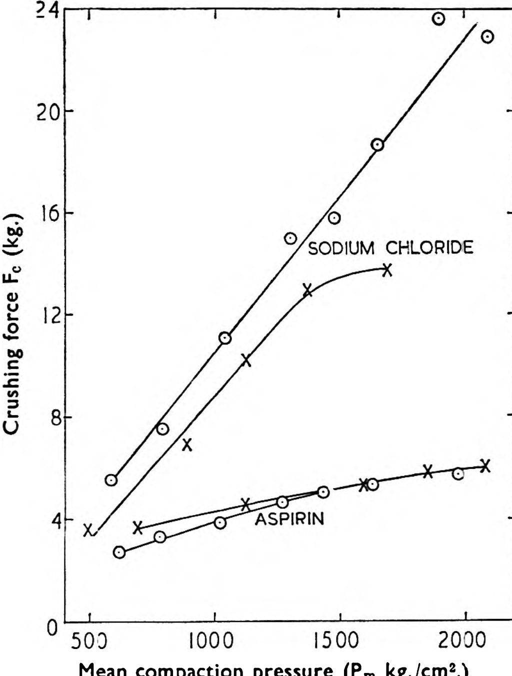

FIG. 7. Relation between crushing force and mean compaction pressure for tablets of aspirin and sodium chloride. × Results from the eccentric machine, ⊙ Results from the rotary machine.

eccentric machine. This is to be expected from the comparison of a compression cycle in which both punches move with a cycle in which the lower punch remains stationary. An average value $P_b/P_a = 0{\cdot}91$ was given during the compression of sodium chloride on a rotary machine. This value remained constant throughout the pressure range under study. On an eccentric machine the ratio P_b/P_a fell from 0·78 to 0·70 as the pressure range was ascended. Similar results were obtained from the compression of aspirin. The punch pressure ratio was slightly lower on both machines, showing a constant value of about 0·89 on the rotary machine and varying from 0·67 to 0·75 on the eccentric machine.

Some of the differences between the value of Pb/Pa for the two machines could be accounted for by difference in die wall polish and in the clearance between punches and die. We think, however, that this effect may be small compared to the effects derived from the different punch movements of the two machines.

Since the upper and lower punch forces recorded on the rotary machine are unequal, the system must be balanced by a force at the die wall acting in an upwards direction and derived from the frictional reaction to a slight downward movement of the tablet during compression. The explanation of this movement was found in the mounting of the lower punch pressure wheel which is depressed during compression, extending an overload spring through a series of levers. Essentially, the tablet is

being compressed between a rigid upper punch and a sprung lower punch.

The consolidation of the materials with increasing pressure is given in Fig. 6. The relation for sodium chloride is almost the same for both rotary and eccentric machines. Thus, the overall density achieved during compression is largely unaffected by the difference in the duration of compression and the inequality of the forces exerted by the upper and lower punches. Aspirin, on the other hand, yielded tablets of lower porosity on the eccentric machine. The difference diminished with increasing pressure and finally disappeared at the highest pressures studied.

Tablets of sodium chloride produced on the rotary machine gave a higher crushing strength than tablets produced at the same pressure on the eccentric machine. The higher strength, as evaluated by the crushing test, may be due to the variation in porosity and therefore strength between the upper and lower parts of the tablet. In tablets produced on an eccentric machine, the porosity and strength will vary markedly in different parts of the tablet because of large differences in the upper and lower punch pressures. It is possible that fracture could be initiated at a lower crushing force, finally indicating a strength which is lower than a tablet of the same density produced on a rotary tablet machine.

The strength of aspirin tablets produced on the two machines was the same at pressures above 1,500 kg./cm.^{2}. In this region a limiting porosity is approached and variations in density and strength in different parts of the tablet will probably be small. At lower pressures, tablets produced on the eccentric machine were slightly stronger. This is commensurate with the lower porosity of these tablets. Comparison of Fig. 6 and 7 show that as the pressure increases, differences in strength and of porosity disappear.

The results from experiments using the feed frame gave pressure ratios similar to the hand-fed series. The wider variation of these results was due to changes in the calibration deflexion during the production of a series of tablets. The lower strength of these tablets was probably due to the production of fine powder as the machine rotated, thus affecting the binding properties of the aspirin.

Acknowledgements. The authors wish to thank Dr. A. C. C. Newman and Mr. D. Stephenson of Burroughs Wellcome Co. Ltd. who kindly made the rotary tablet machine available. The modified transmitter circuits were suggested by the late Mr. J. McCall, formerly of the National Institute of Medical Research, London.

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The paper was presented by PROFESSOR SHOTTON.

THE EVALUATION OF CANADA BALSAM

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Adulteration of commercial samples of Canada balsam with oil is reported, and methods for the detection of this are described. Fresh samples of oleoresin collected during field studies in Quebec Province have been analysed and certain natural variation in the properties of unadulterated oleoresin noted. Acid values may be correlated with the age and condition of trees from which the oleoresins were obtained.

Canada balsam (Canada Turpentine, Balsam of Fir, Gomme de Sapin) is the oleoresin obtained by puncturing blisters which occur on the bark of *Abies balsamea* Mill. The chief industrial use for the oleoresin is for cementing optical lenses, and the adhesive is prepared by dissolving the resin in a suitable solvent. The resin is prepared by removing the volatile oil from the oleoresin with the aid of heat. The resin consists mainly of abietic acid (Lombard, Rotovic and Crigue, 1958; Trost, 1936) with neo-abietic acid and traces of pimaric acids (Lombard and others, 1958). The resene is neutral, inert, of undetermined constitution and is probably a mixture of substances chemically related to the resin acids. The volatile oil contains pinene and phellandrene (Petrowitz, Nerdel and Ohloff, 1962). The resin should be hard and brittle, transparent and pale in colour and have a refractive index near to that of ordinary glass (Bickford and Clark, 1933). During 1961, defective samples of the oleoresin were exported from Canada to Great Britain and the resins prepared from them were soft and sticky, and either opaque, or dark reddish brown in colour. Visual examination of the oleoresins gave little indication that they were defective, although some samples were thinner in consistency than usual. All the samples were investigated on the assumption that variation in properties was due to the presence of another foreign natural product. Fresh oleoresin collected from trees has also been compared with commercial samples.

EXPERIMENTAL

Commercial Samples

As a general test for detecting samples which were not likely to give a hard resin, the following was found useful. Approximately 5 g. of oleoresin was placed in a dish made from aluminium foil and measuring 1·5 cm. deep and 4 cm. diameter. The sample was heated for 18 hr. at 105° in an oven. Each residue was then allowed to cool, and those oleoresins which produced opaque residues or residues too soft to be removed from the foil were regarded as defective. Volatile constituents were determined by this test using a 5 g. sample accurately weighed and heating in an oven at 105° to constant weight. Refractive indices

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and acid values were obtained by the methods of the British Pharmacopoeia 1958. Constituents other than resin acids and volatile oil were determined in a defective sample by the following method. The oleoresin (100 g.) was heated at 105° to constant weight as above, and the soft, opaque residue was dissolved in ether (200 ml.). The resin acids were removed by washing with successive 25 ml. portions of sodium carbonate solution (5 per cent) until the addition of acetic acid to a sodium carbonate extract yielded no further precipitate. The bulked sodium carbonate extracts were washed with ether and the latter added to the original ether extract (A). The combined sodium carbonate extracts were acidified with acetic acid and extracted with ether. The dried ether extract on evaporation gave a solid residue which was weighed. The ether solution (A) was then washed with distilled water, dried over sodium sulphate and evaporated to give an oily residue. This was dissolved in light petroleum (b.p. 60–80°), and filtered to remove traces of insoluble "resene". Removal of solvent gave an oily residue, which was weighed. The refractive index of this oil was determined and an attempt made to saponify the oil using ethanolic potassium hydroxide.

Fresh Samples

These were obtained from four categories of trees. (i) Young trees 8–10 years old of average trunk diameter 10 cm. (ii) Old trees, 47 years old, of average trunk diameter 22 cm., (iii) dead and (iv) diseased trees. Random collections were made from different trees, giving bulk samples of approximately 200 ml. from each source. Acid values, percentage of volatile constituents and yields of resin were determined for each sample as described above.

RESULTS

Commercial Samples

The analytical figures (Table I) include results on oleoresins stored for six years (sample 56/9) and for two years (sample 60/3), also on oleoresins which gave a hard product on drying, and some on adulterated samples. Figures for refractive index were never outside the range 1·520 to 1·523 except for sample 61/2 which gave a result of 1·501. Analysis of this sample yielded resin acids 42·62 per cent and non-volatile

TABLE I

ANALYTICAL RESULTS* FOR COMMERCIAL SAMPLES

Sample	Acid value of oleoresin	Volatile constituents per cent of oleoresin	Yield of resin per cent	Colour and condition of resin	Acid value of resin
60/3	73	28	72	pale yellow, hard	101
56/9	71	29	71	pale yellow, hard	100
61/1	66	25	75	pale yellow, soft	—
61/2	54	20	80	opaque, soft	—
61/3	67	24	76	pale yellow, soft	—
61/4	77	30	70	dark red brown, hard	110
62/1	71	24	76	pale yellow, hard	93
62/2	84	23	77	pale yellow, hard	109

* All based on duplicate determinations.

oil 37·2 per cent. The oil was pale yellow in colour, almost odourless, could not be saponified with alcoholic potassium hydroxide and had a refractive index of 1·459. The resin acids were hard and reddish brown in colour.

When a mixture of good quality oleoresin and liquid paraffin B.P. (33 per cent) was heated at 105° it gave an opaque, soft product; liquid paraffin (5 per cent) gave a soft clear product, whilst one per cent produced a hard resin on drying.

Fresh Samples

The oleoresin from old, diseased and dead trees was thicker in consistency than that from young trees. Resin from young trees was pale yellow, that from diseased trees was cloudy and samples from dead trees were reddish brown. Analytical results are given in Table II.

TABLE II

ANALYTICAL RESULTS FOR FRESH SAMPLES

Sample	Acid value of oleoresin	Volatile constituents per cent of oleoresin	Yield of resin per cent	Colour and condition of resin	Acid value of resin
Young trees	73	25	75	pale yellow, hard	97
Old trees	91	24	76	pale amber, hard	120
Diseased trees	83	26	74	pale amber, hard	112
Dead trees	92	20	80	reddish-brown, hard	115

DISCUSSION

Commercial Samples

The screening test described (see p. 1) not only detects samples adulterated with mineral oils, but will also detect Oregon balsam derived from *Pseudotsgua taxifolia* which gives a soft resin when dried (Bender, 1951). Tests carried out with mixtures of liquid paraffin and oleoresin show that adulteration with 5 per cent mineral oil but not one per cent may be detected by this means. A sample of Canada balsam adulterated with a vegetable oil also gives a soft product after drying but the residue is never opaque and a high saponification value would give corroborative evidence of the presence of glycerides. The opacity of residues obtained from Canada Balsam heavily adulterated with mineral oil is explained by the insolubility of the "resene" in mineral oils: this constituent is consequently deposited when the volatile oil is removed. The "resene" is soluble in vegetable oils, hence the residue from a sample adulterated with such an oil is clear, not opaque. The occurrence of commercial oleoresin having an acid value as low as 54 is new. Previous adulteration was associated with acid values higher than 110 (Roberts and Becker, 1913), and this was due either to substitution with Oregon balsam or crude turpentine. Solvent extraction of sample 61/2 showed that this had been adulterated with what was probably a commercial petroleum oil. From the amount present in the sample, accidental contamination seems unlikely. Visits to the province of Quebec showed that accidental contamination of Canada balsam can easily occur. In place of standard,

clean receptacles, tins used for storing petrol, lubricating oil, heating or cooking oils are often used for collection.

The percentage of volatile constituents gave little indication of the quality of the commercial samples, although gross adulteration with turpentine would be detected by this method. Table I shows that oleoresins stored for several years in a tightly closed container (samples 56/9 and 60/3) are similar to fresh samples from young trees (Table II). The weight of residue after removing the volatile constituents indicates the yield of resin to be expected from good commercial samples only. For adulterated samples, e.g. 61/2, the higher weight of residue is misleading, due to the presence of a liquid non-volatile component. Only one sample (61/2 see p. 2) was shown to be unsatisfactory for optical purposes on the basis of refractive index.

Fresh Samples

Table II indicates a wide range of acid values for the fresh oleoresins: the colour of resin obtained from these samples varies from pale yellow to amber to reddish brown. Acid values of 80–90 (B.P.C. 1934) and 84-87 (Bender 1951) for Canada balsam have been reported, but in the present work, acid values of some good quality commercial samples were 71-77. (Table I). Oleoresins from young trees (8–10 years old) give a lower acid value than oleoresins from older trees (Table II), and in the Montmorency County of Quebec it is from such young trees that resins are obtained. In other regions of Canada where older trees remain, acid values of 80–90 would still be expected. Dead trees would yield acid values in the range 90–100. Since commercial material comprises bulked oleoresins from different sources, however, an average value would be expected. The acid value of Canada balsam also varies with changes in the amount of volatile oil, which is lower in samples from dead trees. Loss of volatile oil (which has an acid value, zero) through defective storage would also cause an apparent increase in acid value. This suggests that an acid value calculated on the basis of the resin content is a more reliable criterion of purity than one calculated in terms of the oleoresin. It is also evident that there would be less likelihood of adulteration of Canada balsam if it were exported and sold in the form of the resin, and this would also reduce transportation costs.

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CHEMICAL STUDIES OF THE LEAVES AND INFLORESCENCES OF *DIGITALIS PURPUREA* L. AND OF ALLIED SPECIES

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The total glycoside content and the nature of the individual glycosides in the leaves and inflorescences of *Digitalis purpurea*, *D. lanata*, *D. lutea*, *D. thapsi* and *D. ambigua* have been studied. A similarity in the chemical composition of the leaves and inflorescences of each species has been demonstrated. The yield of glycosides from *D. purpurea* and from *D. thapsi* would be increased by harvesting the entire aerial parts of the second year plants.

AFTER the discovery of floral organs in commercial samples of the leaves of *Digitalis purpurea* and *D. lanata*, Cowley and Rowson (1958 and 1960), the total glycoside content of the inflorescences of *D. purpurea*, *D. lanata*, *D. lutea*, *D. thapsi* and *D. ambigua*, has been investigated and compared with that of corresponding leaves.

Extensive references are available on the constituents of the leaves of *D. purpurea* and *D. lanata*; less is known however about the constituents of the leaves or inflorescences of any of the species investigated in the present paper.

EXPERIMENTAL AND RESULTS

Material

(i) A commercial sample of first year leaves of *D. purpurea* with a declared potency of 14·26 I.U./g. (ii) First year leaves from wild plants collected at the same time as second year leaves and inflorescences from wild plants. (iii) Second year leaves and inflorescences of the other species were kindly provided by Messrs. Burroughs Wellcome, the Royal Botanic Gardens, Kew and the Botanic Gardens at Oxford and Edinburgh. All samples were dried to constant weight at 55–60°.

Total Glycosides

The first and second year leaves, the inflorescences and the individual morphological members of the inflorescence of *D. purpurea*, also the second year leaves and inflorescences of the other species were assayed for total glycosides. The method used was that of Rowson (1955): the results are shown in Tables I and II.

Individual Glycosides

The nature of the individual glycosides present in the leaves and inflorescences of each species was investigated by the chromatographic technique of Fuchs, Wichtl and Sachs (1958) and Kaiser (1955).

The filtrate (40 ml.), obtained in the total glycoside assay after addition of the sodium sulphate solution (Rowson, 1955), was extracted with chloroform (20 ml.) and then successively with 4 × 20 ml. of a chloroform : methanol (3 : 1) mixture. The combined extracts were dried with

TABLE I

TOTAL GLYCOSIDE CONTENT OF LEAVES AND INFLORESCENCES OF *Digitalis* SPECIES EXAMINED

Species	Source	Total percentage glycosides (calculated as digitoxin)		
		Leaf		
		1st year	2nd year	Inflorescence
D. purpurea	Commercial (14·26 I.U./g.) ..	0·38		
	Wild plants	0·35	0·23	0·33
D. lanata	Messrs. B. W. & Co.	1·02		
„	Kew			0·26
D. lutea	Oxford		0·30	0·21
D. thapsi	Edinburgh		0·80	0·40
„	Messrs. B. W. & Co.		0·76	0·31
D. ambigua	Messrs. B. W. & Co. grown in Kent from—			
	(*a*) French seed		0·47	0·27
	(*b*) Russian seed		0·39	0·17
	(c) German seed		0·91	
„	Oxford		0·40	0·38
„	Kew		0·51	0·26
„	Kew		0·86	0·42

TABLE II

Digitalis purpurea

INDIVIDUAL MORPHOLOGICAL MEMBERS OF INFLORESCENCE (MEANS OF SEVEN DETERMINATIONS)

Total percentage glycoside (calculated as digitoxin)

Flower	Corolla with stamens	Corolla without stamens	Stamens	Calyx with gynaecium
0·45	0·41	0·33	0·78	0·48

Fruit with calyx	Mature calyx	Axis (lower)	Axis (upper)	Seeds	Bract (green)
0·53	0·36	0·20	0·12	0·57	0·49

exsiccated sodium sulphate and filtered. The filtrate was evaporated to dryness below 40° *in vacuo*. The residue was dissolved in the minimum volume of a chloroform : methanol (1 : 1) mixture, transferred to another flask and evaporated to dryness as above. This residue was dissolved in sufficient chloroform : methanol : formamide (2 : 2 : 1) mixture to give an approximately 2 per cent solution of the glycosides, calculated from the assay results already recorded.

The components were run on formamide impregnated paper (Whatman No. 1) using equal parts of xylene and methyl ethyl ketone to separate the secondary glycosides, and chloroform : tetrahydrofuran : formamide (50 : 50 : 6·5) to separate the primary glycosides. Pure reference substances alone and in admixture were also run under the same conditions as the extracts. Ascending solvent technique was used; the temperature was 20 ± 2° and the running time was 3 hr.

After running, the papers were dried at 100° for 1 hr., sprayed with a 25 per cent solution of trichloroacetic acid in ethanol, containing one part in 15 of freshly prepared 3 per cent aqueous chloramine, and dried at 120° for 4 min. They were examined in ultra-violet light.

Results are given in Tables III and IV. Several unidentified spots, belonging to the "A" series of glycosides, were found in the leaves of *D. lutea*, and in the leaves and inflorescences of *D. lanata*, *D. thapsi* and *D. ambigua*.

TABLE III

CONSTITUENTS FOUND IN THE SECOND YEAR LEAVES AND IN THE INFLORESCENCES OF *Digitalis* SPECIES EXAMINED

Constituent	*D. purpurea*		*D. lanata*		*D. lutea*		*D. thapsi*		*D. ambigua*	
	L.	Infl.	L.	Infl.	L.	Infl.	L.	Infl.	L.	Infl.
Purpurea Glycoside A ..	+		+		+		+	+	+	+
Acetyl Digitoxin							+	+		
Digitoxin	+	+	+	+	+		+	+	+	+
Purpurea Glycoside B ..	+	+			+		+	+		
Gitoxin	+	+		+	+	+		+		+
Glucogitaloxin*		+								
Gitaloxin*		+	+		+		+	+		
Gitaloxigenin*		+								
Digitalinum verum	+	+		+		+		+	+	+
Strospeside	+	+	+	+	+	+	+	+	+	+
Lanatoside A			+						+	
Desacetyl Lanatoside C ..			+				+			
Acetyl Digoxin			+							
Digoxin			+							
Lanatoside D			+	+						

* Identified by reference to Kaiser (1955), Van Os (1960) and Witchl (1960).

TABLE IV

CONSTITUENTS FOUND IN THE INDIVIDUAL MEMBERS OF THE INFLORESCENCE OF *Digitalis purpurea*

Constituent	Flower	Corolla with stamens	Corolla without stamens	Stamens	Calyx with gynaecium
Purpurea Glycoside A	+	+	+	+	+
Digitoxin	+	+	+	+	+
Digitoxigenin	+				
Purpurea Glycoside B	+	+	+	+	+
Gitoxin	+	+	+	+	+
Gitoxigenin	+				
Glucogitaloxin*	+	+		+	
Gitaloxin*	+	+	+	+	+
Digitalinum verum	+	+	+	+	+
Strospeside	+	+	+	+	

Constituent	Fruit with calyx	Mature calyx	Axis (lower)	Axis (upper)	Seeds	Bract
Purpurea Glycoside A	+	+	+	+		+
Digitoxin	+	+	+	+		+
Digitoxigenin			+	+		+
Purpurea Glycoside B		+		+		+
Gitoxin	+	+	+	+		+
Gitoxigenin						+
Glucogitaloxin*			+			
Gitaloxin*	+	+		+		
Digitalinum verum		+	+		+	+
Strospeside		+	+			+

* Identified by reference to Kaiser (1955), Van Os (1960) and Witchl (1960).

DISCUSSION

Total Glycosides

Table I shows that the entire inflorescences of *Digitalis purpurea* contained as much total glycoside as did the first year leaves and this was significantly greater than for the leaves of the second year plants.

The morphological members of these inflorescences had a relatively consistent percentage of total glycosides: thus values for the axis were low, and for the stamens, high. For the other members the values were intermediate (Table II). Calcandi and Ciropol (1958) on the other hand found the lowest, not the highest, percentage of glycoside in the stamens.

Of the other species examined only *D. thapsi* and *D. ambigua* (Oxford) had inflorescences with a total glycoside content similar to that of the inflorescence of *D. purpurea*. None of these other species had more glycoside in the inflorescence than in the second year leaves (Table I).

Individual Glycosides

The individual glycosides in the leaves of the species examined showed that *D. lutea* and *D. thapsi* contained mixtures similar to that of *D. purpurea*. The presence of lanatoside A in *D. ambigua* and of derivatives of lanatoside C in *D. lutea* is interesting; the latter observation confirms work quoted by Hoch (1961) which is mostly confirmed by our present observations. The following facts are reported for the first time: purpurea glycoside A and purpurea glycoside B in *D. lutea* and *D. thapsi*; gitoxin in *D. lutea*; gitaloxin in *D. lanata*, *D. lutea* and *D. thapsi*; lanatoside A in *D. ambigua* and strospeside in *D. lutea*, and in *D. thapsi*.

With the exception of *D. lanata* and *D. lutea* the constituent glycosides in the inflorescence were similar to those in the leaf of the same species. It is interesting that primary glycosides of the "A" series were not found in the inflorescences of *D. purpurea*, *D. lanata* and *D. lutea*, although they were found in the morphological members of the inflorescence of *D. purpurea* (Tables III and IV).

The individual glycosides in the inflorescence of *D. purpurea* are the same as those in the leaves: the total glycoside content of these inflorescences is similar to that of first year leaves and is greater than that of second year leaves. It is thus recommended that the official preparation should consist of the dried leaves, or leaves and flowering tops, of *Digitalis purpurea* L.

The inclusion of the inflorescence with the leaves might well apply also to *D. thapsi* and to certain strains of *D. ambigua*.

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The paper was presented by DR. COWLEY.

THE USE OF ORTHOGONAL FUNCTIONS TO CORRECT FOR IRRELEVANT ABSORPTION IN TWO COMPONENT SPECTROPHOTOMETRIC ANALYSIS

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General procedures are outlined for the use of orthogonal functions to correct for irrelevant absorption in two component spectrophotometric analysis. In adapting a traditional method to the use of orthogonal functions, the essential modification occurs at the final stage of calculation, when it is necessary to substitute suitable analogues for the entities, "extinction" and "wavelength." Thus, extinction is replaced by *coefficient of an orthogonal function* and wavelength by *orthogonal function over a specified range (or set) of wavelengths.* Once these substitutions have been made, orthogonal functions may be readily incorporated into the usual methods and equations of spectrophotometric analysis.

Although the procedures are specified in terms of Legendre polynomials (as used by Ashton and Tootill, 1956), they are equally applicable to the use of trigonometric functions, which may have a major role to play in future developments. The above general procedures are illustrated by the analysis of a mixture of adrenaline and phenol.

WHEN properly applied, Vierordt's method gives excellent results in the analysis of a two component mixture and as the result of recent work, the conditions for proper application are now much better understood than they were (Świętoslawska, 1956; Glenn, 1960; Pernarowski, 1961). For the majority of mixtures, accuracies of the order of ± 2 per cent are readily obtainable provided, (i) the absorption curves of the two components are sufficiently different, (ii) a wavelength is available at which the component in question contributes a reasonable proportion of the mixture's total absorption, and (iii) the amount of irrelevant absorption is small. In practice, the last requirement places a considerable restriction upon the application of the method, since the term, "irrelevant absorption," must also include variations of the absorbing impurity content of the components, which occur between batches. Thus, if the mixture has been prepared from batches of material that differ from the "reference" samples used to establish the assay coefficients, the overall effect is equivalent to the introduction of irrelevant absorption. The results suffer accordingly.

In cases, where the general shape of the impurity absorption curve is known, even a cursory comparison usually shows a marked difference in shape between the impurity absorption curve and the curve of the component whose concentration is sought. It is evident that if only one could extract some information from an absorption curve which was fundamentally related to its overall shape, then in most instances, there would be little difficulty in coping with irrelevant absorption. The author's

thoughts on this subject began from this point and in the first instance turned in the direction of harmonic analysis, a standard technique which is regularly used by physicists to characterise curve shapes.

The basis of harmonic analysis is that a given function can be expanded in terms of a set of orthogonal functions (of the same variable, λ). In other words, the function can be broken down into a set of fundamental shapes (orthogonal functions). Thus,

$$f(\lambda) = ag_0 + bg_1 + cg_2 + dg_3 + \ldots \quad \ldots \quad \ldots \quad (1)$$

In the present context, $f(\lambda)$ represents an absorption curve, which according to the above equation can be decomposed into the fundamental shapes, g_0, g_1, g_2, etc., which, except for g_0, are themselves functions of λ. The contribution, which a particular fundamental shape (e.g., g_2) makes to the absorption curve, is given by the appropriate coefficient (e.g., c for g_2). The calculation of such coefficients is greatly simplified by the fact that the set of functions, g_i, are mutually orthogonal, when multiplied together in an integration or matrix process over a specified range of λ. Thus, $g_ig_j = 0$ and if for simplicity, the g_i are normalised, we also have the relationship, $g_ig_i = 1$. Hence, to obtain the coefficient of a particular orthogonal function, g_3, it is only necessary to form the "product", $g_3f(\lambda)$. Having completed this operation, the only non-zero term left on the right hand side of equation (1) is dg_3g_3, which equals d, if the set, g_i, are normalised. As will be mentioned later, these coefficients are proportional to concentration and are equivalent to extinctions (or extinction coefficients where appropriate). In order to minimise errors due to irrelevant absorption, it is of course necessary to choose g_j and also the range of wavelengths so that the corresponding coefficient of the irrelevant absorption is very small relative to that of the component being determined.

In harmonic analysis, the g_i are trigonometric functions but these are slightly less convenient for calculation with a desk machine than are Legendre polynomials, as used by Ashton and Tootill (1956) when dealing with the problem of irrelevant absorption in the assay of griseofulvin in broth. However, although Legendre polynomials are used throughout this paper, there is at present no evidence that in spectrophotometric analysis they are superior to trigonometric functions. This paper is mainly concerned with preliminary thoughts on the practical application of orthogonal functions to the analysis of two component mixtures.

Calculation of Coefficients of Orthogonal Polynomials from Absorption Data

Tables of orthogonal polynomials together with the general method of application are given in standard works on numerical analysis (Milne, 1949; Fisher and Yates, 1953; Davies, 1956). In order to extract from an absorption curve the coefficient of a given polynomial, it is necessary to obtain extinctions at a number of equally spaced wavelengths. For the extraction of the coefficient of a polynomial of a given order, there is a

minimum number of wavelengths which must be used. Thus, to extract the coefficient of the "quadratic" polynomial, P_2, we need a minimum number of three extinctions, namely, E_1 at λ_1, E_2 at λ_2 and E_3 at λ_3, the wavelengths being equally spaced. The coefficient of P_2 is then obtained from the expression, $(+1)E_1 + (-2)E_2 + (+1)E_3$. The numbers in brackets are given in the standard works already mentioned and depend not only upon the order of the polynomial but also upon the number of wavelengths used. For example, the coefficient of P_2 is given by the following expression for 6 equally spaced wavelengths:

$$(+5)E_1 + (-1)E_2 + (-4)E_3 + (-4)E_4 + (-1)E_5 + (+5)E_6$$

whereas for 7 equally spaced wavelengths, it is given by

$$(+5)E_1 + (0)E_2 + (-3)E_3 + (-4)E_4 + (-3)E_5 + (0)E_6 + (+5)E_7$$

For present purposes, there is no need to normalise the coefficients so obtained. The calculation of the coefficients is very easy with a desk calculator and can be greatly simplified by the use of data sheets, so printed that after tabulating the observed extinctions in wavelength order, each extinction lies opposite the number by which it is to be multiplied.

Application of Orthogonal Polynomials to the Analysis of "A" and "B" in a Mixture

Once the coefficients of orthogonal polynomials have been calculated, they can be used in the traditional single and multi-component methods of spectrophotometric analysis just as though they were extinctions (or extinction coefficients, where relevant). The only difference relates to sign, which may be positive or negative in the case of the coefficients of orthogonal polynomials whereas with extinctions, the sign is always positive. *It is of course essential to take account of the sign of the coefficients.*

(1) *The Absorption Curves have Somewhat Different Shapes or are not too Closely Overlapped*

Let P_{it} be a given orthogonal polynomial (e.g., P_3) which is to extend over the particular set of wavelengths, t. From the *E* (1 per cent, 1 cm.) of A at these wavelengths, it is possible to calculate α_{it}, which is the coefficient of P_i for the *E* (1 per cent, 1 cm.) of A at the set of wavelengths, t. If instead of *E* (1 per cent, 1 cm.) of A, we use *E* (1 cm.) of a solution of pure A at concentration, c_A, the coefficient which we obtain by the above process is referred to as μ_{it}. The two coefficients are related by the simple expression:

$$\mu_{it} = \alpha_{it} c_A \qquad \text{Hence, } c_A = \frac{\mu_{it}}{\alpha_{it}}$$

When the solution contains B at concentration c_B, in addition to A, the equation for μ_{it} contains an additional term, $\beta_{it} c_B$.

Hence,
$$\mu_{it} = \alpha_{it} c_A + \beta_{it} c_B \quad \cdots \quad \cdots \quad (2)$$

However, by choosing P_i and the set of wavelengths, t, with sufficient cunning, it may be possible to make the term, $\beta_{it}c_B$, negligibly small. In such a case, the assay for A can proceed as though component B were part of the irrelevant absorption, which is to be eliminated by the process of calculating μ_{it}. It may also be possible to choose another polynomial, P_j, and/or another set of wavelengths, u, so that c_B can be evaluated whilst component A is ignored.

These procedures are really limiting cases of two basic methods given in section (2). Thus, the methods suggested in this section are the same as those which receive detailed treatment in section (2), except that c_A and c_B do not have to be evaluated by means of simultaneous equations.

Before proceeding further, it is worth stressing the fact that the above equations have just the same form as the traditional equations of spectrophotometric analysis. For example:

$$E\,(1\text{ cm.})_{\lambda_1} = E\,(1\text{ per cent, }1\text{ cm.})_{A\lambda_1}c_A \qquad \text{("traditional")}$$

$$\mu_{it} = \alpha_{it}c_A \qquad \text{("orthogonal")}$$

It is evident from this pair of equations that the "μ" coefficients of the present treatment correspond to E (1 cm.) values, whereas the "α" and "β" coefficients correspond to extinction coefficients. This analogy is a general one so that any of the equations of spectrophotometric analysis can be adapted to the use of orthogonal functions by writing "coefficient of an orthogonal function" in place of "extinction". Furthermore, there is a similar general analogy between "orthogonal function over a specified range (or set) of wavelengths" and "wavelength". Hence, suffixes such as "it" that occur in the present treatment have the same significance as suffixes which denote wavelength in the traditional equations.

To sum up: orthogonal functions can be applied to any of the traditional methods of spectrophotometric analysis provided that, (i) "coefficient of an orthogonal function" be substituted for "extinction" and, (ii) "orthogonal function over a specified range (or set) of wavelengths" be substituted for "wavelength."

(2) *The Absorption Curves have Similar Shapes and Overlap Considerably*

Purely for the sake of simplicity, it is assumed throughout this section that all polynomials are of the "four point" variety. In practical applications, it may be necessary to use "higher point" polynomials (e.g., 12 point). However, the following theory would only alter in respect to the number of wavelengths specified. A given mixture may be tackled by two general methods.

(*a*) *Using Two Sets of Wavelengths, "t" and "u"*

Two polynomials, P_{it} and P_{ju} are required, each of which refers to a particular set of wavelengths. Unlike (2*b*) below, there is no need for "i" and "j" to be different. Thus, both polynomials could be P_2.

To obtain c_A and c_B, we require the experimental data listed in Table I. λ_1 to λ_4 constitute the wavelength set, "t," whilst λ_5 to λ_8 constitute the set "u".

TABLE I

NECESSARY EXPERIMENTAL DATA WHEN USING FOUR POINT POLYNOMIALS IN CONJUNCTION WITH TWO SETS OF WAVELENGTHS, "t" AND "u"

Data	Wavelengths at which data is required								Polynomial coefficients to be obtained
E (1 cm.) of mixture	λ_1	λ_2	λ_3	λ_4	—	—	—	—	μ_{it}
	—	—	—	—	λ_5	λ_6	λ_7	λ_8	μ_{ju}
E (1 per cent, 1 cm) of A	λ_1	λ_2	λ_3	λ_4	—	—	—	—	α_{it}
	—	—	—	—	λ_5	λ_6	λ_7	λ_8	α_{ju}
E (1 per cent, 1 cm) of B	λ_1	λ_2	λ_3	λ_4	—	—	—	—	β_{it}
	—	—	—	—	λ_5	λ_6	λ_7	λ_8	β_{ju}

From Table I, it is evident that the mixture's E (1 cm.) values at λ_1, λ_2, λ_3, and λ_4 are used to calculate μ_{it}, the "mixture" coefficient of the polynomial, P_{it}. In the same way, the mixture's E (1 cm.) values at λ_5, λ_6, λ_7, λ_8, are used to calculate μ_{ju}, the "mixture" coefficient of the polynomial, P_{ju}. The other coefficients are obtained similarly as indicated in Table I.

c_A and c_B are then evaluated from the following pair of simultaneous equations:

$$\mu_{it} = \alpha_{it}c_A + \beta_{it}c_B$$

$$\mu_{ju} = \alpha_{ju}c_A + \beta_{ju}c_B$$

(b) Using One Set of Wavelengths, "t," Throughout

Two *different* polynomials, P_i and P_j, are required (e.g., P_2 and P_3). To obtain c_A and c_B, we require the experimental data given in Table II.

TABLE II

NECESSARY EXPERIMENTAL DATA WHEN USING FOUR POINT POLYNOMIALS IN CONJUNCTION WITH ONE SET OF WAVELENGTHS, "t"

Data	Wavelengths at which data is required				Polynomial coefficients to be obtained
E (1 cm.) of mixture	λ_1	λ_2	λ_3	λ_4	μ_{it} and μ_{jt}
E (1 per cent, 1 cm.) of A	λ_1	λ_2	λ_3	λ_4	α_{it} and α_{jt}
E (1 per cent, 1 cm.) of B	λ_1	λ_2	λ_3	λ_4	β_{it} and β_{jt}

From Table II, it is evident that the mixture's E (1 cm.) values at λ_1, λ_2, λ_3, λ_4, are used to calculate, (i) μ_{it}, the "mixture" coefficient of the polynomial, P_i, (ii) μ_{jt}, the "mixture" coefficient of the polynomial, P_j. The "α" and "β" coefficients have similar meanings, which can be seen from the same Table.

c_A and c_B are then evaluated from the following pair of simultaneous equations:

$$\mu_{it} = \alpha_{it}c_A + \beta_{it}c_B$$

$$\mu_{jt} = \alpha_{jt}c_A + \beta_{jt}c_B$$

Experimental

An experimental trial of each method discussed in sections (1) and (2) was carried out using a mixture of adrenaline (0·0033 per cent w/v) and phenol (0·0060 per cent w/v). The solvent was aqueous 0·1N H_2SO_4 throughout. Extinctions were measured on a Uvispek photoelectric spectrophotometer, great care being taken with the setting of the wavelength scale. The absorption curves of adrenaline, phenol and the mixture of the two are shown in Fig. 1. The results are shown in Table III.

TABLE III

Experimental results

Assay	Wavelength range (mμ)	Intervals (mμ)	Polynomial	Percentage recoveries	
				Adrenaline	Phenol
I	264–297	3	P_3	not determined	99·2
II	241·5–296·5	5	P_3	95·8 (98·7)	not determined
III	252–285	3	P_2	101·8	97·8
	264–297	3	P_3		
IV	270–292	2	P_2 and P_3	101·0	99·4

Twelve point polynomials were used throughout, this being a compromise between (i) the need to extract as much data as possible from the (continuous) absorption curves, and, (ii) the analytical labour involved.

Discussion of Results

Assays I and II (Table III) exemplify the determination of just one component by the selection of a polynomial and set of wavelengths for which the second component makes a negligible contribution to the "mixture" coefficient (see section (1)). The poor result obtained for assay II was due to the fact that over the chosen range of wavelengths, the phenol P_3 coefficient deviates significantly from zero upon even a minute change of the starting wavelength. It is not therefore a good choice, but when allowance was made for the known small (non-zero) value of the phenol P_3 coefficient, the result improved to the recovery quoted in brackets. Assay III exemplified the method described in section (2*a*), whilst assay IV was an example of the method described in section (2*b*).

The above results are encouraging and suggest that the use of orthogonal functions warrants a careful study over a wide field of applications. The main source of error in these results is believed to reside in the setting of the wavelength scale, since above 270 mμ, the phenol absorption curve

has a particularly steep slope. In order to accumulate the extinction data that is needed to calculate the coefficients, μ, α and β, three solutions have to be measured at each of the chosen wavelengths. This can be done by measuring either, one solution at a time over the whole set of wavelengths, or, three solutions in succession at each wavelength. The second alternative eliminates wavelength setting errors entirely, for it is then possible at each wavelength to obtain the necessary extinction data from all three solutions without disturbing the wavelength scale. However, it was inconvenient to adopt such a procedure on the author's instrument.

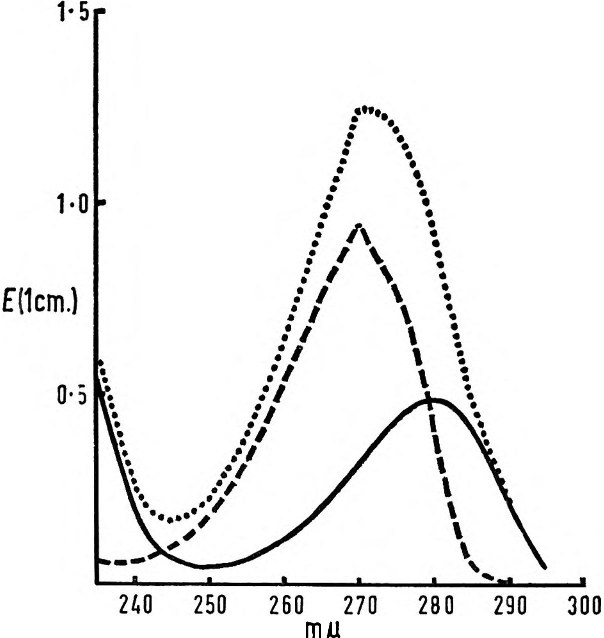

Fig. 1. Ultra-violet absorption curves of adrenaline and phenol in 0·1N aqueous H_2SO_4. —— = Adrenaline (0·0033 per cent w/v). ---- = Phenol (0·0060 per cent w/v). ..... = Adrenaline (0·0033 per cent w/v) + Phenol (0·0060 per cent w/v).

The same mixture of adrenaline and phenol was also assayed by the modified Vierordt method (Glenn, 1960) using the wavelengths 270 mμ and 283 mμ. The recoveries were (*a*) 100·6 per cent for adrenaline and (*b*) 98·9 per cent for phenol. However, had this mixture contained a linear irrelevant absorption such that the extinction of the mixture was increased by one quarter at 270 mμ and one third at 283 mμ, the recoveries would have been, (*a*) 137·8 per cent for adrenaline and (*b*) 118·2 per cent for phenol. Nevertheless, the results obtained by the use of orthogonal functions would not have been altered in any way by the same linear irrelevant absorption, which if present would produce such catastrophic results in the modified Vierordt method.

An assay based on orthogonal functions rejects all components of the irrelevant absorption curve other than those which are used to calculate the assay coefficients. ("Component" is used in the mathematical sense throughout this paragraph.) Thus, in assays I and II, the P_3 component of the irrelevant absorption is the only one that is not eliminated, which means in particular that the constant, linear and quadratic components of the irrelevant absorption are all rejected. Assay III on the other hand rejects the constant, linear and all higher components of the irrelevant absorption but not the quadratic, P_2, component, for this was used to calculate the assay coefficients. It follows that the general procedure described in section (2*b*), which requires the use of two different polynominals over one set of wavelengths, is inherently less able to correct for irrelevant absorption than are the other methods. For example, in assay IV, the P_2 and P_3 components of the irrelevant absorption are not eliminated. Nevertheless, it is probable that on average, irrelevant absorption contributes much more to the constant and linear components of the total absorption than to any others and these are of course eliminated by any of the procedures outlined above.

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Short Communication

CHARGE DELOCALISATION IN RELATION TO NEUROMUSCULAR BLOCKING ACTIVITY OF CERTAIN TETRAALKYLAMMONIUM COMPOUNDS

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Holmes, Jender and Taylor (1947) related the curariform activity of certain quaternary compounds to the charge delocalisation in the molecule. The amount of charge delocalisation is dependent on the electronegativity of the alkyl groups linked to the central nitrogen atom. They postulated that if the density of charge on the central atom fell below a critical level, the compound would be pharmacologically inactive at the neuromuscular junction. Their evidence was based on dissociation constants of aliphatic amines and carboxylic acids. It is well known that the ionisation constants of bases are dependent not only on the inductive effect, but also on the steric effects of those substituents. The dissociation constants of carboxylic acids would seem to reflect most closely the inductive effects of alkyl substituents.

Holmes and his colleagues were limited by lack of reliable values for the dissociation constants required and by their scant pharmacological data. They used neuromuscular blocking potencies taken from the work of Ing and Wright (1934) who measured the time for complete paralysis of frog sartorius muscles. Further work was clearly indicated.

Our work uses cats and the method, depending on the depth rather than the rate of onset of paralysis, would seem more reliable in relation to this theory. An attempt has been made to extend the studies of Holmes and others and to determine the relationship, if any, between the charge delocalisation and pharmacological activity at the neuromuscular junction, for a series of six simple quaternary ammonium compounds.

Method

Contractions of the left gastrocnemius muscle of spinal atropinised cats were recorded using a Brown-Schuster myograph. The sciatic nerve was stimulated by supramaximal rectangular pulses (6 per min., 0·5 msec. duration). Retrograde injections were made into the right iliac artery so that drugs were carried with the blood stream down the left leg.

Drugs. The compounds studied were tetramethylammonium iodide (TMA), tetraethylammonium iodide (TEA), tetra-n-propylammonium iodide (TPA), tetra-n-butylammonium iodide (TBA), tetra-n-amylammonium bromide (TAA) and tetra-n-hexylammonium bromide (THA). TMA and TEA were obtained from commercial sources; TPA, TBA, TAA and THA were isolated and purified by Dr. E. R. Clark in this laboratory. All doses are expressed in terms of the chemical base.

RESULTS AND DISCUSSION

After intra-arterial injection of doses up to 12 mg. of drug, all the compounds except TEA interfered with neuromuscular transmission in the cat (Fig. 1); TEA was inactive in these doses. Results from four experi-

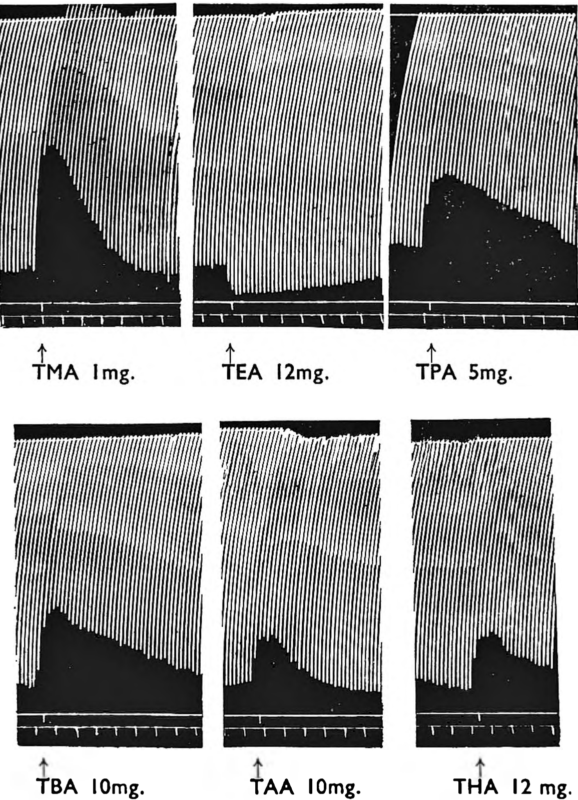

FIG. 1. Cat, spinal, atropine. Contractions of left gastrocnemius muscle in response to sciatic nerve stimulation (6 shocks/min., 0·5 msec. duration). Drugs injected into the right iliac artery as indicated. Time signal, 60 sec.

ments clearly show the order of potency to be TMA > TPA > TBA > TAA > THA > TEA. Table I shows K_a values for the appropriate carboxylic acids (Ingold, 1953).

TABLE I

THERMODYNAMICALLY CORRECTED ACIDITY CONSTANTS FOR CERTAIN CARBOXYLIC ACIDS. (INGOLD, 1953)

Acid	Group – R of R–COOH	$K_a \times 10^5$
Acetic ..	.. CH_3–	1·75
Propionic	.. C_2H_5–	1·33
n-Butyric	.. C_3H_7–	1·50
n-Valeric	.. C_4H_9–	1·38
Caproic ..	.. C_5H_{11}–	1·32
n-Heptylic	.. C_6H_{13}–	1·28

Stronger acids (i.e., with higher K_a values) will reflect lower electropositivity (+I effect) for the alkyl group and therefore the order of +I is hexyl > amyl > ethyl > butyl > propyl > methyl. The greater the +I value, that is the greater ease with which they yield electrons towards an electron-attracting centre, the lower will be the density of charge on the central nitrogen atom in the corresponding tetra-alkylammonium compound. Therefore, according to the theory of Holmes and others, the order of pharmacological potency should be TMA > TPA > TBA > TEA > TAA > THA. Except for TEA this was the order of neuromuscular blocking activities found in this study. Of this series of compounds only TEA antagonises neuromuscular blocking agents (Collier and Exley, unpublished). This action of TEA is presynaptic in origin (Stovner, 1957) and probably involves the release of acetylcholine. It is likely that this presynaptic effect would reduce the blocking action of TEA on the postsynaptic membrane. Thus the exceptionally low neuromuscular blocking activity of TEA is not surprising.

Though there are undoubtedly many other factors influencing the biological activity of these compounds, the experimental results in cats support the theory that concentration of charge favours their neuromuscular blocking activity.

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The paper was presented by MR. COLLIER.

Short Communication

THE REACTION OF SEMICARBAZIDE WITH COLLAGEN

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When experimental lathyrism is induced in rats by administering certain aminonitriles or hydrazine derivatives two major effects observed are increased fragility and increased extractability of collagen in skin and other connective tissues (Levene and Gross, 1959). Abundant collagen is synthesised in the skin of lathyritic rats but it does not mature in the same way as in normal animals, as judged by comparing the sub-unit composition of neutral-salt-soluble and acid-soluble collagens from normal and lathyritic animals (Martin, Gross, Piez and Lewis, 1961; Martin and and Goldhaber, 1963; Martin, Piez and Lewis, 1963). Moreover, although collagen fibrils can be reconstituted, *in vitro*, from solutions of neutral-salt-soluble collagen of lathyritic rats, they are less stable than those formed in a similar way from the neutral-salt-soluble collagen of normal rats (Gross, 1963). It has been suggested that lathyritic agents act by altering the aggregation stage of collagen fibrogenesis (Smiley, Yeager and Ziff, 1962; Gerber, Gerber and Altman, 1962; Witschafter and Bentley, 1962), possibly by inhibition of cross-linking reactions (Martin and others, 1961, 1963). Evidence has been put forward for the direct action of lathyritic agents on collagen during maturation (Levene, 1962; Martin and Goldhaber, 1963). This paper reports preliminary observations on the effect of treating collagen *in vitro* with one lathyritic agent, semicarbazide (Levene, 1961). Its effect on fibril forming properties and sub-unit composition has been measured.

Neutral-salt-soluble collagen was extracted from the skins of 4–6 week old rats with 1·0M sodium chloride and purified (Wood, 1962). Solutions (0·1 per cent) of this material in 0·1M sodium chloride adjusted to pH 4·1 with 0·005M acetate buffer (Wood and Keech, 1960) were treated for known times at 35° with an equal volume of 0·15M phosphate buffer at pH 7·0 and 1/5 volume of 0·5M semicarbazide hydrochloride adjusted to pH 7·0 with sodium hydroxide. In control mixtures 0·5M sodium chloride was substituted for the semicarbazide solution. The reaction mixtures were then dialysed exhaustively against 3 per cent acetic acid and finally against 0·1M sodium chloride adjusted to pH 4·1 as above.

Fibril formation. The kinetics of fibril formation at 35°, and pH 7·0, in treated and control solutions were followed turbidimetrically (Wood and Keech, 1960). The results, of which those shown in Fig. 1 are typical, showed that treatment with the lathyritic agent retarded fibril formation. The effect was most marked on the lag period or nucleation phase (Wood, 1960). When the precipitates were cooled to 0–4° a fraction redissolved (Gross, 1958; Fessler, 1960) but the rate at which this occurred was approximately the same in treated and untreated solutions.

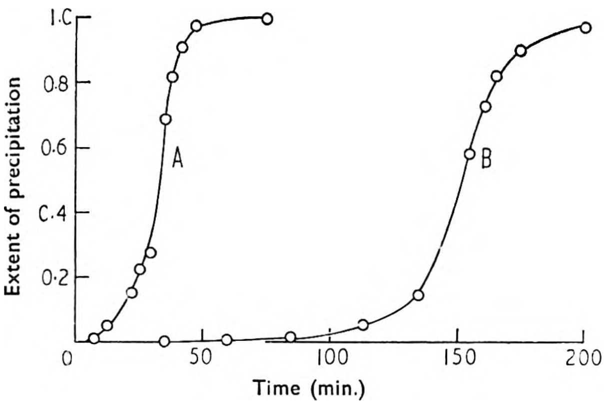

FIG. 1. Rate of precipitation at pH 7.1, 35°, I 0.23, of fibrils in solutions (0.1 per cent) of (A) neutral-salt-soluble collagen and (B) neutral-salt-soluble collagen treated with semicarbazide for 3 hr.

Sub-unit composition. Fibril formation was allowed to occur as above in solutions of treated and untreated collagen at 35° and pH 7·1 and the precipitates "aged" at 35° for three weeks (using toluene as preservative). The collagens were isolated, dissolved in 3 per cent acetic acid and dialysed against 0·15M acetate buffer, pH 4·8. The solutions (approximately 0·25 per cent) were denatured by heating to 45–50° and examined in the ultra-centrifuge at 40° (Wood, 1962). A sample of untreated neutral-salt-soluble collagen which had not been "aged" at 35° was also examined and showed the two major components (α and β) described by others (Orekhovich and Shpikiter, 1955) in relative proportions (Table I) close to those observed earlier (Wood, 1962). Both semicarbazide-treated and untreated samples which had been "aged" also showed these components but contained in addition appreciable quantities of a third, faster moving, component. The apparent relative proportions of these components (Table I) show two interesting features; (i) as suggested by earlier work (Wood, 1962) the relative proportions of the components change on ageing, the concentration of α falling, the concentrations of β and the third component rising; (ii) a similar shift is observed in "aged" semicarbazide-treated collagen but the magnitude of the effect appears to be slightly less than in the untreated sample.

TABLE I

Material	Composition (g./100 g.)		
	α	β	Third component
Neutral-salt-soluble collagen (NSS)	78	22	trace
"Aged" NSS	59	25·5	15·5
"Aged," semicarbazide-treated, NSS	63	28	9

Discussion. The results show that direct action of semicarbazide on neutral-salt-soluble collagen *in vitro* resulted in changes in the collagen somewhat similar to those observed in lathyrism. The treated collagen

formed fibrils less readily than untreated collagen although the fibrils formed were not markedly less stable than with untreated collagen. The change of sub-unit composition observing on "ageing" neutral-salt-soluble collagen may be relevant to the similar change which occurs in maturation of collagen *in vivo* and which is believed to be due to cross-linking of the polypeptide chains of the protein. In lathyrism the collagen synthesised is not as highly cross-linked as in normal animals. The present data suggest that the cross-linking, which seems to occur during "ageing," does not proceed as readily in semicarbazide-treated collagen as in untreated collagen.

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The paper was presented by THE AUTHOR.

AN EXPERIMENTAL DETERMINATION OF THE INTER-NITROGEN DISTANCE IN SOME BIS-QUATERNARY AMMONIUM GANGLIONIC AND NEUROMUSCULAR BLOCKING AGENTS

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The conductances in water of hexamethonium bromide and iodide, decamethonium bromide and iodide, hexadecamethonium iodide, and decaethonium iodide have been measured. The values of the limiting cationic conductances have been interpreted in terms of molecular shape, and the internitrogen distance for the above compounds calculated, using hydrodynamic relationships. The internitrogen distances found were 6·3 Å for hexamethonium, 9·5 Å for decamethonium, 13·5 Å for hexadecamethonium, and 10·2–13·5 Å for decaethonium. The effect of the hydrocarbon/water interfacial energy on the extended lengths of these molecules is discussed.

WHEN organic ions having aliphatic hydrocarbon chains as part of their structure are in aqueous solution, there is a considerable interfacial energy between the hydrocarbon chain and the solvent. The organic part of the molecule will contract as much as possible to minimise the interfacial area and thus the interfacial energy. The amount of contraction for mono- and di-carboxylate ions, trimethylalkylammonium ions, and dodecylamine and dodecyl sulphate ions has recently been calculated (Elworthy, 1963), and it has been shown that the amount of contraction depends on the length of the aliphatic chain, and on the number of ionised groups present in the molecule. Many neuromuscular and ganglionic blocking agents contain aliphatic hydrocarbon chains, and would be expected to show such contraction. As the internitrogen distance is one of the important quantities in developing theories of the mode of action of this type of drug, then clearly it is important to determine the distance for the molecule as it exists in aqueous solution, rather than in the dry state. Calculations of the inter-nitrogen distance have been made by Gill (1959) for ganglionic blocking agents. In this paper an experimental method for determining this distance is reported.

Theoretically, transport properties can be interpreted in terms of molecular shape by applying Perrin's (1934, 1936) relationships between the frictional resistance and the shape of the molecule. This is an approach familiar in macromolecular chemistry. For ions with a radius below 5 Å, difficulties in interpretation are encountered as Stokes' Law fails in this region of molecular size. Recently however, Perrin's relationships have been shown to apply to small molecules (Elworthy, 1962), and following a method based on that suggested by Stokes and Robinson (1959a), deviations from Stokes' law have been assessed, a procedure involving the determination of volumes and hydrations of ions (Elworthy, 1963). From the limiting equivalent ionic conductance λ^0, the molecular shape can be obtained.

The balance between the driving force on an ion, and its frictional resistance leads to the following equation (Stokes and Robinson, 1959b)

$$\lambda^0 = |z|\, F^2/Nf \qquad (1)$$

$|z|$ being the valency, f the frictional coefficient, N is Avogadro's number and F the Faraday. For spherical ions, Stokes' Law gives f in terms of the ionic radius, r, and the viscosity of the medium, η. Asymmetric ions are represented by models, usually ellipsoids, and for such a model the mean frictional coefficient is

$$f^o_e = 12\pi\, \eta/S \qquad (2)$$

giving

$$\lambda^o_e = |z|\, F^2.S/(12\pi.\eta N) \qquad (3)$$

S is a function of the three semi-axes of the ellipsoid, a, b, and c. Prolate ($a > b = c$) and oblate ($b > a = c$) ellipsoids of revolution are usually chosen to represent molecules, avoiding the indeterminacy of interpreting an experimental quantity in terms of three unknowns. The molecules studied here fit best into the prolate model for which:

$$S = (2/\sqrt{a^2-b^2})\ \ln.\ [(a + \sqrt{a^2-b^2})/b)] \qquad (4)$$

knowing the volume of the ellipsoid, $V = 4\pi ab^2/3$, a and b can be obtained. The above equations can also be used in terms of the ratio f^o_e/f^o_o ,where f^o_o is the frictional coefficient of a sphere of the same volume as the ellipsoid. The ratio a/b can be found from the value of f^o_e/f^o_o, either using Perrin's equation (4) or from tables (Svedburg and Pedersen, 1940).

A method for assessing the volumes of organic ions for this type of hydrodynamic calculation has been developed, being a procedure roughly similar to the determination of the closest distance of approach of water molecules to the ion (Elworthy, 1963). Stokes and Robinson (1959c) suggest that quaternary ammonium ions are generally unhydrated in solution.

EXPERIMENTAL

Materials. A sample of hexamethonium bromide was converted to the iodide by releasing the free base with a strong anion exchanger, and neutralisation with A.R. hydriodic acid. Decamethonium iodide and bromide were gifts of Allen & Hanburys, Ltd., while decaethonium iodide was supplied by Professor Stenlake. Hexadecamethonium iodide was prepared by treating 1,16-diiodohexadecane with trimethylamine in ethanol. All samples were recrystallised at least three times from suitable solvents, and assayed by converting a weighed amount into free base or free acid by passage over the relevant strong ion-exchanger, and titrating the effluent with standard acid or alkali.

Sodium and potassium chloride A.R. were recrystallised twice from conductance water, and dried at 250°. Water twice distilled, had a specific conductance $\sim 1 \times 10^{-6}$ mhos.

Measurements. The conductance bridge was an LKB 3216B high precision model, used in conjunction with a Cambridge low inductance six decade box as a measuring resistance. The conductance of the water

was determined on the same day as the solutions made from it, using a cell with cell constant, $k = 0{\cdot}102_5$. The solutions were made up by weight, and measured in a cell, $k = 0{\cdot}952_2$. The cells were extensively calibrated with solutions of potassium chloride, and checked by measuring the conductances of sodium chloride solutions. Agreement to within $\pm$ 0·1 per cent of the literature figures for the latter solutions was obtained. During measurement, the cells were placed in an oil thermostat controlled to 25 $\pm$ 0·003°.

Results and Discussion

The conductance results are shown in Figs. 1 and 2 as plots of equivalent conductance (Λ) against (normality)$^{\frac{1}{2}}$. Preliminary Λ^0 values were found by extrapolating the phoreograms to zero concentration.

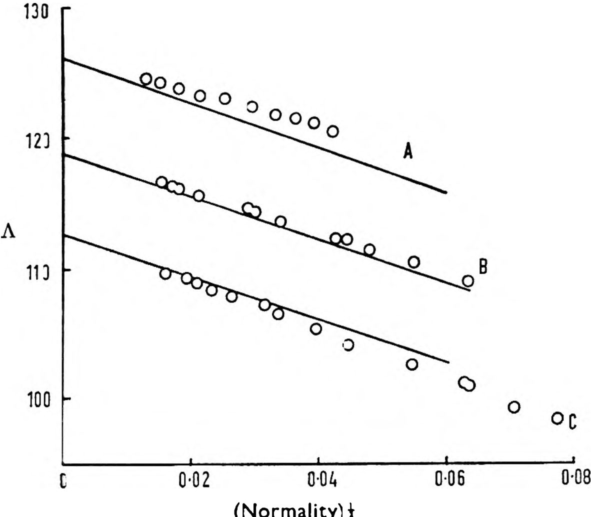

Fig. 1. Plots of equivalent conductance (Λ) against (normality)$^{\frac{1}{2}}$ for A, hexamethonium iodide. B, decamethonium iodide. C, hexadecamethonium iodide.

The hexa- and decamethonium salts approach the Onsager limiting tangent from above, which is the general criterion for a non associated electrolyte. The results were fitted using the Fuoss-Onsager (1957) equation.

$$\Lambda = \Lambda^0 - Sc^{\frac{1}{2}} + Ec \log c + Jc \quad \ldots \quad \ldots \quad \ldots \quad (5)$$

where c is the concentration in equivalents per litre, E and J are constants.

The Onsager limiting tangent is shown in the Figures as a full line. Hexadecamethonium and decaethonium iodide both approached the limiting tangent from below, thus showing some measure of association in solution. As the precision of the results was only $\pm$ 0·1 per cent, the full scale analysis based on the extended law was not undertaken. The method of Fuoss and Shedlovsky gave a reasonable means of determining Λ^0 (see Fuoss and Accascina, 1959).

Using 78·1 for λ^0 (Br^-) and 76·8 for λ^0 (I^-), the λ^0 values for the cations were:

	λ^0 from bromide	λ^0 from iodide
Hexamethonium	49·3	49·4
Decamethonium	42·0	42·1
Decaethonium	—	35·5
Hexadecamethonium	—	35·9

We are concerned only with the interpretation of the limiting ionic conductance in terms of molecular shape, using hydrodynamic methods. The other method of approach is that of Rice (1958) which represents the chain as a set of spheres joined by links, imposing restrictions due to hindered rotation about bonds, interactions between charged groups, etc. In Rice's method, as in Gill's calculations, the effect of the hydrocarbon/water interfacial energy has been neglected whereas in the present method, the model used takes this effect into account.

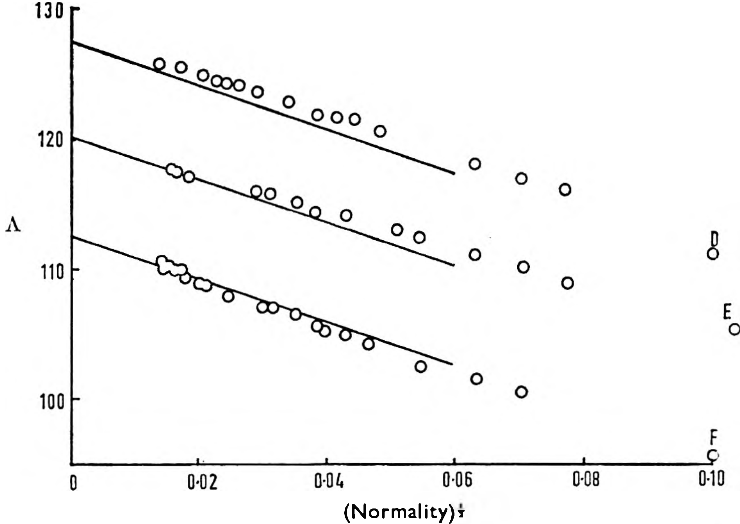

FIG. 2. Plots of equivalent conductance (Λ) against (normality)½ for D, hexamethonium bromide. E, decamethonium bromide. F, decaethonium iodide.

Using the observed λ^0 values, and the correction procedure for Stokes' Law previously described (Elworthy, 1963), a and b for prolate ellipsoids representing the molecules were found. The model chosen to fit the molecule is an ellipsoid equivalent to it, and the positions of the centres of the nitrogen atoms in this equivalent ellipsoid were estimated by the following procedure.

Integration to find the volume of half the ellipsoid leads to

$$\tfrac{1}{2}V = \frac{\pi b^2}{a^2}\left[a^2x - \frac{1}{3}x^3\right]_0^x \quad \ldots \quad (6)$$

x being a distance along the major axis to the chosen point. Knowing a, b, and the volume equivalents of each group in the molecule, the values

of x corresponding to the volume of the molecule inside and outside the nitrogen atom can be found from (6). Hence the distance between the centres of the nitrogen atoms can be found.

	Hexa-methonium	Deca-methonium	Hexadeca-methonium	Deca-ethonium
N^+–N^+ distance (Å)	6·3	9·5	13·5	10·2–13·1

For decaethonium there are several possible arrangements of the ethyl groups on the nitrogen atoms. These groups can lie alongside the main part of the hydrocarbon chain, or point outwards in the x direction. To minimise the total hydrocarbon/water interfacial energy, it would be expected that as many groups as possible would lie in the first position, giving 13·1 Å for the internitrogen distance. With two ethyl groups in close proximity to the main hydrocarbon chain, and one directed outwards, 11·5 Å is obtained; while with one group close to the chain, and two directed outwards 10·2 Å is found. The remaining arrangement does not seem possible, as molecular models show that there is insufficient space on one side of a nitrogen atom to accommodate all three ethyl groups. The internitrogen distances found are larger than that of decamethonium. The results reflect that a measure of protection against the contracting effect of the hydrocarbon/water interfacial energy is given to the hydrocarbon chain by introducing large substituents on the nitrogen atom.

In previous work the degree of extension of molecules containing the same hydrocarbon chain was, dicarboxylic acids > monoalkyl trimethylammonium compounds > monocarboxylic acids. The present series of bistrimethylammonium compounds gives a slightly greater degree of extension than the dicarboxylic acids; due to the repulsive forces between the polar groups, which oppose the contracting effect of the interfacial energy, and possibly due to the "protecting" effect of the paraffinoid groups attached to the quaternary nitrogen atoms. This effect was clearly shown in the greater extension in solution of monoalkyltrimethylammonium compounds as compared with monocarboxylic acids.

The internitrogen distance found for hexamethonium (6·3 Å) is slightly shorter than the mean internitrogen distance calculated by Gill (6·9 Å) but falls within the range of his probability calculations, e.g. between 6 and 7·8 Å; it is about 3 Å shorter than that measured on a Catalin model arranged in a regular configuration. The additional reduction found here is due to the interfacial energy effect discussed above. At the moment no attempt at detailed discussion of potential attachment points on receptor surfaces will be made, as conductance measurements on other ganglionic and neuromuscular blocking agents are required.

For decamethonium, the N^+–N^+ distance of 9·5 Å is some 4 Å shorter than the Catalin distance, but is in accord with previous suggestions of 9–10 Å (Carey, Edwards, Lewis, and Stenlake, 1959). It is interesting to note that substitution of ethyl for methyl groups on the nitrogen atom increases the internitrogen distance (though this effect will only occur if the chain linking the nitrogen atoms is a flexible one). Peak activity in the bistriethylammonium series occurs in the tridecyl compound (Barlow and Ing, 1948), which would have an internitrogen distance of between

12 and 15 Å, a value roughly interpolated from a graph of N^+–N^+ distance against the number of carbon atoms joining the nitrogen atoms. This value greatly exceeds that of 9·5 Å found for decamethonium, which has peak neuromuscular blocking activity in the methonium series. Although internitrogen distance is an important factor in relation to activity, many others have to be considered.

Acknowledgements: I thank Professor J. B. Stenlake and Mr. J. J. Lewis for reading the manuscript, and Miss M. Buchanan for technical assistance.

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The paper was presented by THE AUTHOR.

ON THE RELATIONSHIP BETWEEN THE EFFECT OF PHENOL ON THE OXYGEN UPTAKE AND THE VIABILITY OF *PENICILLIUM NOTATUM* SPORES

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0·05 to 0·2 per cent phenol in Horowitz medium progressively reduces the oxygen uptake of *Penicillium notatum* spores. 0·08 per cent causes fungistasis and fungicidal action is evident at 0·15 per cent and higher concentrations. Exogenous respiration is less sensitive to phenol than spore germination. Measurement of oxygen uptake can not be used for quantitative evaluation of fungicidal action. The concentration exponent for the effect of phenol on oxygen uptake is $-1{\cdot}5$.

THE respiration of cells—which includes all cellular oxidations yielding energy—is usually measured in terms of oxygen uptake or carbon dioxide formed. There is relatively little information correlating the effects of fungicides on the respiration and viability of fungal spores. It was, therefore, of interest to measure the respiration of the spores of *Penicillium notatum* in the presence and absence of phenol and to relate it, if possible, to the fungistatic and fungicidal action.

EXPERIMENTAL

The methods of preparation of spore suspensions containing $37{\cdot}5 \times 10^6$ spores per ml. and of evaluation of fungicidal activity were those of Chauhan and Walters (1961).

The procedure for measurement of oxygen uptake was essentially that described by Umbreit, Burris and Stauffer (1959), using a Warburg apparatus at 28° and a shaking speed of 100 oscillations per min. To the body of each reaction flask was added 1·5 ml. of double-strength Horowitz (1947) fluid medium (pH 5·3), 0·5 ml. of freshly prepared spore suspension and sufficient sterile water to produce 3 ml., allowing for the volume of 0·5 or 1·0 per cent phenol solution added to the sidearm. The centre well contained 0·2 ml. of 20 per cent potassium hydroxide solution and a 2 cm. square of fluted filter paper. The phenol solution was tipped into the body of the reaction flask, usually 1 hr. after closing the manometer tap, but in some cases after 4, 6 or 9 hr. Readings were taken hourly for 12 hr. and at intervals for a further 12 hr., where practicable. Duplicate reaction flasks and thermobarometers were set up for each experiment and all results are the means of replicate experiments.

To determine whether there was fungistasis a reaction flask was opened after 12 or 24 hr. and a sample of the suspension examined microscopically for swollen spores and germ tubes. Absence of bacterial contamination was confirmed at the same time. The suspension was then filtered, the spores washed, and fungicidal activity evaluated; counts were made after 4 to 48 hr. incubation, according to the phenol concentration and time before addition.

RESULTS

Fig. 1 shows that the oxygen consumption of *P. notatum* spores in Horowitz medium decreased as the phenol concentration was increased from 0·05 to 0·2 per cent; 0·25 per cent produced but little further reduction. Spore germination was not inhibited by 12 hr. contact with 0·05 per cent phenol. In 0·06 per cent phenol large swollen spores were present after 12 hr. and most of these produced germ tubes in 24 hr. Spores were

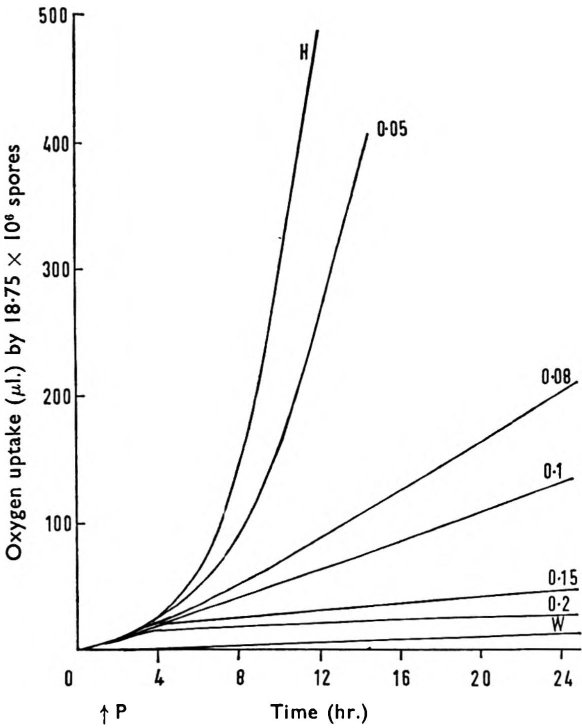

FIG. 1. Oxygen uptake of *P. notatum* spores at 28° in Horowitz medium (H) and with phenol added (P ↑) after 1 hr. to give a concentration of 0·05—0.20 per cent, and in water (W).

only slightly swollen in 0·08 per cent phenol after 12 hr. and there was no change after 24 hr. Above this concentration there was neither swelling nor germination in 24 hr. Less than 20 per cent of spores were killed in 12 hr. by 0·05 to 0·15 per cent phenol, a much higher percentage was killed with higher concentrations or a longer (24 hr.) contact time (Table I).

Fig. 2 shows that the rate of oxygen uptake per hr. increased in the absence of phenol. When phenol was added after 1, 4, 6 or 9 hr. to give a concentration of 0·1 per cent, the rate remained almost the same as that before addition but with higher concentrations it decreased markedly with increase in the time before addition. The fungicidal action was much greater when phenol was added after 4 hr. than after 1 hr. but there

TABLE I

EFFECT OF CONCENTRATION OF PHENOL ON OXYGEN UPTAKE AND VIABILITY OF *P. notatum* SPORES

Phenol* concentration (per cent)	Oxygen uptake (µl.) by 18·75 × 10⁶ spores in		Germination† in		Per cent survivors after	
	12 hr.	24 hr.	12 hr.	24 hr.	12 hr.	24 hr.
0	486	—	+	+	95	—
0·05	273·6	—	+	+	95	—
0·06	151·9	—	−S	±S	89	—
0·08	94·2	205·8	−S	−S	89	83
0·1	61·6	132·0	—	—	86	91
0·13	39·5	66·9	—	—	87	—
0·15	32·4	47·1	—	—	80	38
0·176	27·0	33·4	—	—	57	—
0·2	19·7	27·2	—	—	37	13
0·25	18·0	26·0	—	—	14	0·002
Control	7·0	12·3	—	—	95	97

* Spores suspended in Horowitz medium and phenol solution added after 1 hr. to give concentration shown. Control = spores in water only.

† Suspension examined for germination and swelling (S) of spores, then filtered, spores washed and incubated in Horowitz medium to determine per cent survivors. (+ = all spores germinated, ± = many germinated, — = none germinated).

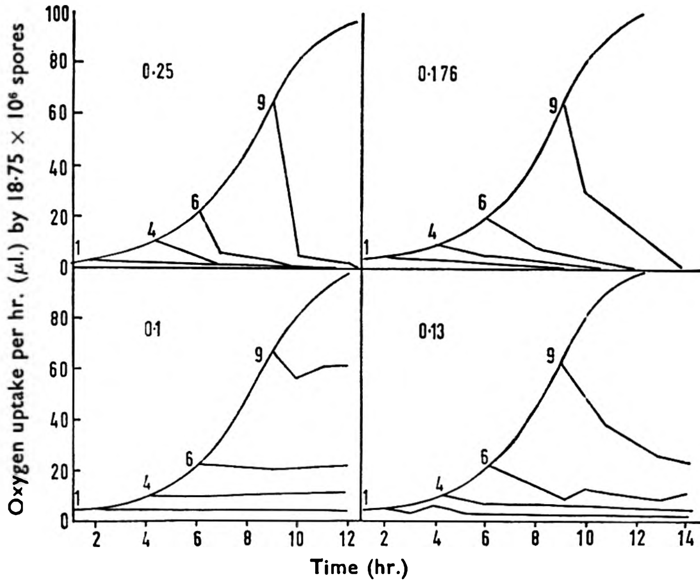

FIG. 2. Effect of adding phenol after 1, 4, 6 and 9 hr. to give a concentration of 0·1, 0·13, 0·176 or 0·25 per cent on the oxygen uptake of *P. notatum* spores in Horowitz medium at 28°.

was little further increase in activity when it was added after 6 or 9 hr. (Table II).

Spores in Horowitz medium without phenol showed no change in size after 1 hr. but after 4 hr. they were swollen and after 6 hr. about 10 per cent had produced small germ tubes. All spores appeared to have germinated in 9 hr. The production of germ tubes was accompanied by an increased rate of oxygen uptake (Figs. 1 and 2).

TABLE II

EFFECT OF TIME OF ADDITION OF PHENOL ON THE VIABILITY OF *P. notatum* SPORES

Phenol* concentration (per cent)	Per cent survivors after 12 hr.† when phenol was added after			
	1 hr.	4 hr.	6 hr.	9 hr.
0·1	88	83	79	63
0·13	87	68	65	67
0·15	80	32	30	27
0·176	57	4	1	<1
0·25	15	c. 0·007	c. 0·001	c. 0·001

* Spores suspended in Horowitz medium and phenol solution added after 1, 4, 6 or 9 hr. to give concentration shown.
† Suspension filtered 12 hr. after commencement, spores washed and incubated in Horowitz medium to determine per cent survivors.

DISCUSSION

The mean results of duplicate manometric experiments carried out on different occasions with and without phenol, were satisfactory when compared by the *t* test (Table III). For 10 different results the mean oxygen uptake of spores in Horowitz medium in 12 hr. was 486 μl. with a coefficient of variation of 4·2 per cent, indicating good reproducibility.

TABLE III

RESULTS OF COMPARISON OF OXYGEN UPTAKE DURING 12 HR. BY *P. notatum* SPORES IN HOROWITZ MEDIUM AND WITH ADDED PHENOL

Phenol concentration (per cent)*	0	0·05	0·06	0·08	0·10	0·13	0·15	0·176	0·2	0·25
t	0·163	0·095	0·772	1·669	0·943	0·257	1·597	1·184	0·057	1·448
P	0·8–0·9	>0·9	0·4–0·5	0·1–0·2	0·3–0·4	0·7–0·8	0·1–0·2	0·2–0·3	>0·9	0·1–0·2

* As for Table I.

The ratios of exogenous to endogenous oxygen uptakes in 6, 9 and 12 hr. were 38·7, 40·8 and 69·4 respectively, showing that Horowitz medium is a sensitive growth medium for examining the effect of phenol on the oxygen uptake and viability of *P. notatum* spores. The addition of increasing concentrations of phenol to the growth medium progressively *reduced* these ratios until in Horowitz medium containing 0·2 per cent phenol they were 8·5, 3·4 and 2·8. The opposite effect has been noted and McCallan, Miller and Weed (1954) report that the oxygen consumption of the spores of *Neurospora sitophila*, *Aspergillus niger* and some other fungi was *increased* by phenol and other fungicides. Fig. 3 shows that the log-log relationships are linear between phenol concentrations of 0·05 to 0·2 per cent and oxygen uptake by *P. notatum* spores in 9, 12 and 24 hr.: they are also linear between concentrations of 0·05 to 0·1 per cent, and times (from Fig. 1) for an uptake of 120 or 200 μl. The calculated slopes of the lines in Fig. 3B are 1·3 and 1·7 giving a mean concentration exponent of −1·5 for the effect of phenol on oxygen uptake.

Absence of germination in 0·08 per cent phenol in 24 hr. shows that this is the minimum fungistatic concentration. A large amount of oxygen is, however, taken up during this period, indicating that inhibition of

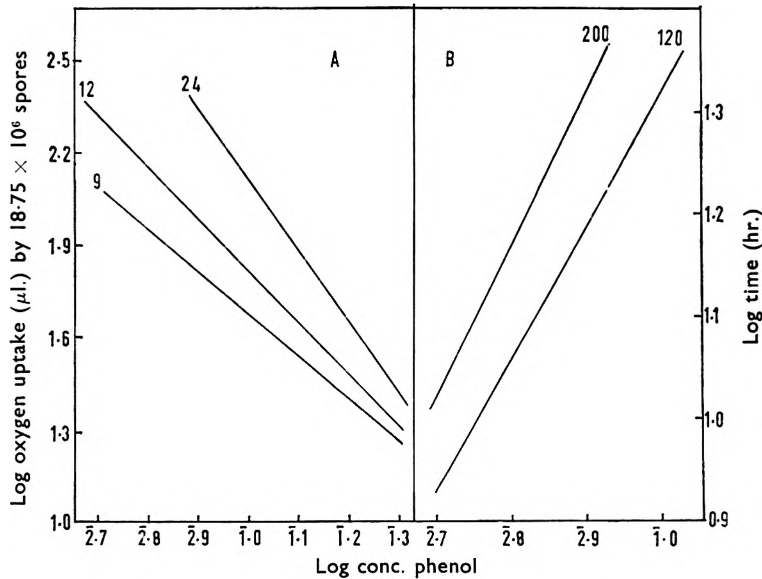

FIG. 3. (A) Relationship between log oxygen uptake in 9, 12 and 24 hr. by *P. notatum* spores and log phenol concentration. (B) Log time—log phenol concentration relationship for oxygen uptakes of 120 and 200μl. by *P. notatum* spores.

germination is a more sensitive criterion of the toxicity of phenol than is inhibition of respiration. A similar conclusion for different fungi and fungicides has been reported whereas claims have been made that inhibition of oxygen uptake parallels growth inhibition (see Cochrane, 1958).

Although oxygen uptake is considerably reduced by fungicidal concentrations of phenol, the change in uptake with concentration is very small compared with the change in the viable spore population. Oxygen uptake thus cannot be used as a quantitative measure of the fungicidal activity of phenol and in this respect our views agree with those of McCallan and others (1954). It should be noted, however, that with bacteria, correlation between bactericidal activity and reduction of oxygen uptake has been reported (Sykes, 1958).

Swollen and germinated spores are more susceptible to phenol than unswollen spores. This is presumably because the former have cell walls which are more permeable to nutrients and hence also to phenol.

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The paper was presented by DR. WALTERS.

Short Communication

HEAT AND GAMMA-RADIATION RESISTANCE OF *BACILLUS MEGATERIUM* SPORES

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The experiments reported are part of a series designed to investigate whether or not the heat resistance of bacterial spores can be correlated with resistance to other inimical physical agents such as ionising or ultra-violet radiation and dehydration.

It has been shown that heat resistance is affected by the divalent metallic ion content of growth and sporulation media (Slepecky and Foster, 1959; Black, Hashimoto and Gerhardt, 1960), and an investigation of this effect was the starting point for the present work.

A chemically defined liquid medium containing Mg and Fe as the sole divalent metallic ions was formulated for the production of spores of *Bacillus megaterium* (ATCC8245). It is referred to as "GS" medium and consists of K_2HPO_4 0·3 g., KH_2PO_4 0·1 g., NH_4Cl 0·05 g., NH_4NO_3 0·01 g., Na_2SO_4 0·01 g., $MgSO_4{\cdot}7H_2O$ 0·001 g., $FeSO_4{\cdot}7H_2O$ 0·0001 g., glucose 0·1 g., L-glutamic acid and L-asparagine·H_2O 10^{-2} M, water to 100 ml.; the pH is adjusted to 7·0–7·2. The general procedure for spore production is as follows: a drop of a standard spore suspension is added to 20 ml. of GS medium. This culture is grown aerobically at 37° until the appearance of filamentous cells and then subcultured into a second 20 ml. of GS medium. Aerobic incubation of the subculture is carried out for 36 hr.; sporulation is by then complete. The spores are washed three times in sterile water, resuspended in water and stored at 4°.

Samples of such a suspension were heated for different periods in a bath at 100° and survivors estimated from colony counts. Exponential time/survival curves were constructed and the slopes of these used as a measure of heat resistance, the greater the value of the slope the less resistant are the spores to heat. Similarly, slopes of exponential dose/survival curves were used as a measure of gamma-radiation resistance. Exposures to cobalt-60 gamma-rays were carried out at 22° with spores suspended in aerated water.

The GS medium was modified by substituting Mn and/or Ca for Fe, and also by adding Mn or Ca or both. This gave batches of spores with different resistances to heat.

We find: (1) Mg is essential for sporulation, together with Fe or Mn. No spores are obtained if Fe is substituted by Ca alone; (2) spores produced in GS medium (Mg and Fe) are the least resistant to heat, slope = 0·14 sec.$^{-1}$; (3) substitution in the GS medium of Fe by Mn plus Ca gives spores with about twice this resistance to heat, slope = 0·060 sec.$^{-1}$; (4) addition of Ca to GS medium has no effect on heat resistance, slope = 0·15 sec.$^{-1}$; (5) addition of Mn to GS medium gives spores with

an intermediate heat resistance, as does the addition of both Mn and Ca, slopes of both batches 0·092 sec.$^{-1}$; (6) the gamma-radiation resistance is unchanged by these changes in the divalent metallic ion content of the GS medium, slopes between 0·014 and 0·012 Krad^{-1}.

These preliminary results confirm earlier reports that the high resistance of spores to heat depends to some extent on the divalent metallic ion content of the sporulation medium. At this stage the specific roles of these ions in sporulation and in regulation of heat resistance cannot be recognised, but clearly they are not similarly involved in regulation of gamma-radiation resistance. However, we cannot as yet reject the concept that these resistances are correlated. It should be noted that of the spores produced by these techniques, those with the lowest heat resistance are many times more heat resistant than the corresponding *B. megaterium* vegetative cells. It may well be that an unidentified mechanism responsible for this disparity is a common regulatory mechanism for the usual high resistance of spores to both heat and radiation.

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The paper was presented by DR. TALLENTIRE.

THE EVALUATION, FROM EXTINCTION DATA, OF THE INACTIVATION OF BACTERIOPHAGE BY CHEMICAL AGENTS

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The inactivation of coliphage T6r by phenol has been examined using an extinction method and the reproducibility of the results shown to be acceptable. Two methods of interpreting the results are described and their relationship discussed. A linear relationship has been found between log extinction time and log phenol concentration for extinction times between 2 and 200 min.

MANY investigations of the inactivation of phages by chemical agents have been made using plaque counting methods.

These studies have been mainly concerned with the dynamics of the inactivation process but not with complete inactivation, i.e. sterilisation.

A few investigations (Hunter and Whitehead, 1940; Klein, Kalter and Mudd, 1945; Deutsch and Rohr, 1955) have used end-point or extinction methods but the procedures gave extinction time estimates of low precision and were used for qualitative comparisons of the viricidal activity of the agents.

Extinction methods have been widely used with antibacterial agents but many of the methods can be criticised as giving extinction time estimates which lack precision and reproducibility. One exception is when the analysis devised by Mather (1949) is applied to the results obtained by methods similar to that described by Berry and Bean (1954). This method has been adapted for the investigation of the viricidal action of phenol and other chemical agents on a coliphage.

EXPERIMENTAL

Bacterial host. The bacterial host was a laboratory strain of *Escherichia coli* which was sensitive to the phage. The organism was grown at 37° in peptone water (1·0 per cent Oxoid peptone and 0·5 per cent sodium chloride in distilled water; pH 7·0) or on peptone agar (peptone water with 2½ per cent shredded agar). Cultures were maintained on peptone agar slopes stored at room temperature and subcultured monthly. Cultures in peptone water were initiated by subculture from a stock slope and maintained by daily serial subculture for up to 6 days; the cultures were normally used after 18 to 30 hr. incubation. In peptone water cultures the viable count reached 4×10^8 organisms per ml. in 18 hr. without aeration and 4×10^9 organisms per ml. with active aeration, the counts being performed by the surface viable method of Miles and Misra (1938).

Bacteriophage. The phage used was a coliphage obtained from the School of Hygiene and Tropical Medicine, London. It had the cultural

characteristics of the strain classified as T6 by Demerec and Fano (1945) in the "r" (rapidly lysing) form described by Hershey (1946). The phage was cultivated by a modification of the method described by Hershey, Kalmanson and Bronfenbrenner (1943) in which peptone agar plates were surface seeded with a mixture of *E. coli* and phage in a ratio which gave barely confluent lysis when the plaques were fully developed. The phage particles were harvested by flooding each plate with 3 ml. of sterile distilled water, allowing them to stand for 20 to 30 min., decanting the suspension formed and removing bacteria and debris by centrifuging (7,000 r.p.m. for 20 min.) and filtering the supernatant through 5/3 sintered glass. This procedure gave 2·2 to 2·6 ml. of phage suspension per plate; the phage titre varying from 6 to 9 $\times$ 10^{10} infectious particles per ml. In addition to the phage, the lysates contained peptone and sodium chloride extracted from the solid medium and soluble degradation products from the host cells. The solid content of the lysates, determined by evaporation to constant weight over silica gel, was 1·54 per cent and the sodium chloride content, by the assay of the British Pharmacopoeia, 1958, was 0·50 per cent. The same values were obtained for sterile peptone water and the lysates were therefore considered to be equivalent to suspensions of phage in peptone water.

All phage inocula used were taken from a single stock suspension stored at 5°. Plaque counts performed on the stock at intervals gave a mean count of 4·06 $\times$ 10^{10} phage particles per ml. with 95 per cent confidence limits of $\pm$0·29 $\times$ 10^{10}. The method of plaque counting used was based on that described by Williams-Smith (1951, 1953) and details of the modifications used will be reported in a subsequent communication.

The constancy of titre, plaque morphology, host specificity and rate of lysis of broth cultures of the host were accepted as evidence of absence of significant variation in the phage.

Apparatus. For the measurement of small volumes, dropping pipettes were used as described by Cook and Yousef (1953). When calibrated gravimetrically the pipettes were found to deliver a mean single drop volume of 1/58 ml. of water and 1/63 ml. of peptone water. The 95 per cent. confidence limits of the mean volume of 6 drop samples was approximately 0·2 per cent of the mean. Automatic tilt measures* were used to measure 20 ml. volumes of media. The volume delivered by these measures, as determined by weighing 10 separate volumes of water delivered by each of 3 measures, was 19·98 $\pm$ 0·04 ml. (P = 0·95).

Solutions of Phenol, Analar, were prepared immediately before use by diluting 5 per cent stock solutions which were stored in the dark for up to one month. All solutions were prepared using sterile distilled water.

Inhibition of Phage Growth

A serial tube dilution method was used to determine the bacteriostatic concentration of phenol for the bacterial host in peptone water and the

* "E-mil Brand", Kipps measures, manufactured by H. J. Elliott, Ltd., Glamorgan

rate of mass lysis in phage cultures containing sub-bacteriostatic concentrations of phenol. The bacteriostatic tests were performed in triplicate and each culture was inoculated with approximately 4×10^6 viable cells per ml. of culture medium. In the phage inhibition tests, 5 replicate cultures were prepared for each concentration of phenol tested and inoculated with approximately 4×10^6 viable host cells and 1×10^4 phage particles per ml. of culture. Both types of culture were incubated at 37° and their opacities periodically compared visually with those of control cultures containing no phenol. The phage tests were repeated using cultures with phage inocula averaging $1{\cdot}5 \times 10^8$ and 0·15 particles per ml. of culture and containing the maximum concentration of phenol previously shown to permit an approximately normal rate of growth of the host.

Method of Evaluating Phage Inactivation

Ten ml. of the phenol solution under test was pipetted into a glass-stoppered tube of approximately 20 ml. capacity and placed in a water-bath at 25 ± 0·05° for 30 min. to allow the temperature of the solution to equilibrate with that of the bath. The phenol solution was then inoculated with 6 drops (0·095 ml.) of the phage suspension from a dropping pipette and the mixture immediately shaken vigorously for 20 sec. Approximately 1 ml. of the mixture was drawn into a sterile dropping pipette and 6-drop samples of it delivered into a number of empty sterile test-tubes whose temperature had previously been equilibrated at 25° by storage in the bath for 30 min. and which were immediately returned to the bath. Normally 9 to 11 such samples were distributed.

The time of contact of the phage with the phenol was measured from the moment the first drop of phage inoculum entered the solution. At suitable intervals of time each reaction tube was withdrawn from the bath and 20 ml. of peptone water added to it from a flask fitted with an automatic tilt measure. After mixing, 6 drops of an 18–25 hr. peptone water culture of *E. coli* were added from a dropping pipette, the tube was again shaken and placed in a water-bath maintained at 37°. The tubes were incubated at 37° and their opacity compared with that of control cultures after 3 to 4 hr., 18 to 24 hr., 2 days, 3 days and 4 days. The control cultures contained the same concentration of phenol and received the same host inoculum as the diluted reaction mixtures but contained no phage.

The presence of surviving infective phage particles was shown by visible lysis in the incubating cultures. The reliability of the periodic examination of the cultures in detecting active phage was confirmed by placing loopfuls of reaction mixture cultures which had been incubated for 4 to 7 days, on the surface of peptone agar plates previously surface seeded with host cells. On incubating these plates, a uniform lawn of bacterial growth was produced except where loopfuls of culture had been placed. There, a more dense region of bacterial growth resulted but, where the culture contained active phage, a clear halo of lysis surrounded this region.

RESULTS

Inhibition of Phage Growth

The host strain of *E. coli* grew in the presence of 0·2 per cent but not in 0·25 per cent phenol. However, in 0·2 per cent phenol and all concentrations tested down to 0·025 per cent the rate of growth was slower than in the absence of phenol. Using phage cultures, mass lysis occurred in all concentrations of phenol permitting growth of the host cells but in those concentrations giving slow host growth, lysis was correspondingly delayed. No evidence of the selective inhibition of the phage was found. In 0·025 per cent phenol, lysis occurred at a normal rate and infection of the cultures by the smallest phage inocula used could be detected. In these cultures, mass lysis was obvious after 7 hr. incubation and their opacity remained less than that of the control host cultures for at least 26 hr. In the cultures with larger phage inocula, lysis was obvious within 2 hr. but after 12 hr. incubation their opacity equalled that of the control host cultures.

Phage Inactivation

In most of the reaction mixture cultures containing active phage, lysis was apparent after 18 to 24 hr. incubation. An earlier examination was necessary to detect lysis in those cultures where large numbers of phage had survived exposure to the phenol. In such cultures, lysis of the sensitive host cells and growth of the resistant cells was rapid and after 18 hr. incubation their opacity equalled that of the control cultures. The inspections at 2, 3 and 4 days were made since a number of cultures showed lysis only after 2 days incubation. In some experiments the cultures were examined at daily intervals for up to 7 days but in no case was lysis obvious after longer than two days incubation.

The result of a single test for the inactivation of the phage has been expressed as the Inactivation Time which is the time of contact of phage and phenol after which no active phage can be detected in the reaction mixture. A minimum of 5 replicate determinations was considered necessary to give a reliable estimate of the Inactivation Time, the mean of the individual tests being calculated and expressed as the Mean Inactivation Time (M.I.T.). In any one group of replicate tests the time intervals at which the samples of reaction mixture were diluted were selected so that, within the 9 to 11 samples taken from each reaction mixture, at least one sample contained active phage and at least the last two samples diluted show no phage activity. Provided these conditions were satisfied the time intervals were made as short as possible so as to achieve maximum precision in the M.I.T. estimates. Each M.I.T. determination involved a number of preliminary experiments to obtain an approximate value. The required distribution of samples containing active phage was found in most instances with time intervals of approximately 1/8 to 1/10 of the expected M.I.T.

A typical set of results, obtained from three separate determinations of the M.I.T. of 3 per cent phenol for the phage, are shown in detail in Table I.

TABLE I

THE DETERMINATION OF THE MEAN INACTIVATION TIME (M.I.T.) OF 3 PER CENT PHENOL FOR COLIPHAGE T6r AT 25°

Experiment	Replicate test	Contact time (min.) 16	20	24	28	32	36	40	44	48	Inactivation time (min.)
I	1	+	−	+	−	−	−	−	−	−	28
	2	+	−	+	+	−	−	−	−	−	32
	3	+	−	+	−	−	+	−	−	−	40
	4	+	+	+	−	+	−	−	−	−	36
	5	+	+	+	+	−	−	−	−	−	32
	6	+	+	−	−	−	−	−	−	−	24
										M.I.T.	192/6 = 32·0
II	1	−	−	−	+	−	−	−	−	−	32
	2	+	−	−	−	−	−	+	−	−	44
	3	+	+	+	+	+	−	−	−	−	36
	4	+	+	−	+	−	−	−	−	−	32
	5	+	+	−	−	−	−	−	−	−	24
	6	+	+	−	+	−	−	−	−	−	32
										M.I.T.	200/6 = 33·3
III	1	+	+	+	+	+	−	+	−	−	44
	2	+	+	+	+	−	−	−	−	−	32
	3	+	+	−	−	−	−	−	−	−	24
	4	+	+	+	−	+	−	−	−	−	36
	5	+	+	+	+	−	−	−	−	−	32
	6	+	+	+	+	−	+	−	−	−	40
										M.I.T.	208/6 = 34·7

+ = active phage present; − = no phage activity.

Reproducibility of the Method

The level of confidence with which the M.I.T. can be taken as an estimate of the inactivation of phage by phenol is shown by the results of a series of replicate determinations of the M.I.T. for five different concentrations of phenol. These experiments are summarised in Table II.

The individual estimates of the M.I.T. for each concentration of phenol are the results of separate experiments carried out at various times during a period of 3 months.

TABLE II

THE MEAN INACTIVATION TIME (M.I.T.) OF VARIOUS CONCENTRATIONS OF PHENOL FOR COLIPHAGE T6r (25°)

Concentration of phenol (per cent w/v)	Contact time interval (min.)	Number of replicate tests in each determination	Range of inactivation times (min.)	M.I.T. (min.)	Mean M.I.T. (min.)	95 per cent confidence limits of mean M.I.T. (min.)
3·50	½	5	2½–4 2½–4 2½–4	3·04 3·20 2·75	3·00	±0·57
3·25	1	5	9–13 9–12 8–11	10·8 10·6 9·4	10·3	±1·80
3·00	4	6	24–40 24–44 24–44	32 33 35	33·3	±3·79
2·90	5	5	25–60 30–60 40–50	47 50 45	47·3	±6·25
2·66	20	5	180–240 180–240	204 208	206	±25·4

Estimation of the Mean Single Survivor Time

The results of the experiments referred to in Table II were subjected to the analysis devised by Mather (1949) which permits the calculation of the Mean Single Survivor Time (M.S.S.T.) and the standard error of the estimations. The M.S.S.T. is defined as the time at which there is, on the average, one surviving organism per sample volume. The analysis of the results is summarised in Table III.

TABLE III

ESTIMATION OF MEAN SINGLE SURVIVOR TIMES (M.S.S.T.) FROM THE REGRESSION BETWEEN TIME OF CONTACT AND LOG (− LOG PROPORTION OF NEGATIVE SAMPLES). COLIPHAGE T6r EXPOSED TO PHENOL AT 25°

Phenol concentration (per cent w/v)	Visual estimate of M.S.S.T. (min.)	Calculated estimates			
		First approximation		Second approximation	
		M.S.S.T. (min.)	Standard error	M.S.S.T. (min.)	Standard error
3·50	2·03	2·12	0·18	2·12	0·17
3·25	7·45	7·25	0·39	7·35	0·36
3·00	23·60	23·16	1·22	23·33	1·06
2·90	36·75	36·54	2·09	36·75	2·06
2·66	124·5	119·6	15·5	121·2	14·0

Correlation between M.I.T. *or* M.S.S.T. *and Concentration of Phenol*

The regressions of log M.I.T. and log M.S.S.T. against log phenol concentration were analysed by standard statistical methods as described by Brownlee (1949). The correlation coefficients were 0·9985 and 0·9988 respectively with 3 degrees of freedom and the correlations were therefore highly significant. The calculated regression coefficient for log M.I.T. against log phenol concentration was −15·140 and the residual variance about the regression line was 0·002. For log M.S.S.T. against log phenol concentration, the corresponding values were −14·659 and 0·001. The variance of the difference between the regression coefficients was 0·383 from which $t = 0{\cdot}777$ with 6 degrees of freedom which corresponds to a probability between 0·4 and 0·5.

A linear relationship therefore exists both between log M.I.T. and log phenol concentration and between log M.S.S.T. and log phenol concentration. The regression lines representing these relationships are parallel over the time range of 2 to 200 min., and the mean ratio of M.I.T. to M.S.S.T. over this time range is 1·439.

DISCUSSION

Any study of the inactivation of phage by a chemical agent must include an investigation of the possible inhibition, by the agent, of the growth of the host and of the phage in cultures of the host. It is then essential that, when testing for phage surviving at the end of the inactivating process, the agent is neutralised chemically or is diluted to a concentration which not only will permit the growth of the host cells but also permit the infection of the host by small numbers of phage particles. This point does not appear to have received the attention it merits in previous investigations.

In devising an extinction time technique for use with phage, two other problems arise, that of lysis of the host by inactivated phage and multiplicity reactivation of the phage. The test applied to each sample of the reaction mixtures at the end of an inactivation period was intended to show simply whether or not infective phage had survived. The number of host cells added to the diluted reaction mixture samples was approximately equal to the number of phage originally present in the sample. While the cultures were examined at relatively infrequent intervals, cultures from samples giving increasing time of contact of the phage with a given concentration of phenol showed, in general, an increase in lysis time corresponding to that to be expected from decreasing numbers of phage in the inocula. The tests therefore gave no indication that inactivated phage particles were adsorbed to and lysed the host cells or that multiplicity reactivation occurred.

A very few reaction mixture sample cultures showed a pronounced delay in lysis, clearing of the culture being apparent only after 2 days incubation. The phenomenon was rare and occurred only in cultures from samples quenched at times approaching the final inactivation time, when the numbers of infective particles present were small. In these cultures, the rate of lysis was much slower than that shown by small phage inocula in the presence of sub-bacteriostatic concentrations of phenol and no explanation of the effect can be offered until more data is available.

In the present experiments, simple dilution of the reaction mixture samples was relied upon to overcome inhibition of the growth of the host or surviving phage. The reaction mixture samples were made relatively large to minimise sampling errors and the concentrations of phenol required to inactivate the phage were high in relation to the concentrations inhibiting its growth. The volumes of peptone water used to dilute the samples were, therefore, larger than those commonly used in bacterial extinction tests.

The phage titre of the inoculum was standardised by plaque counts but the survival of the phage exposed to phenol was estimated by its ability to grow in fluid culture. Justification for this comparison can be found in the close agreement previously shown between plaque counts and dilution end-point counts (Hershey and others, 1943; Kleczkowski and Kleczkowski, 1951).

To obtain a precise estimate of the inactivation time of the phage by various concentrations of phenol the contact time interval used in the tests was short in relation to the final inactivation time. A wide variation was found, however, between individual estimates of the inactivation times, the range between maximum and minimum values obtained from 5 to 6 replicate estimations commonly being from 50 to 75 per cent of the mean estimate. This variation is an indication of the distribution of resistance to inactivation between individual phage particles in the phage inoculum. The use of a 5 or 6 fold level of replication was found to give a mean estimate of the inactivation time which adequately reflected the distribution of resistance and the reproducibility of which was therefore

acceptable. In Table II, the 95 per cent confidence limits of the mean M.I.T.'s are about 10 to 20 per cent of the mean. It should be noted that the estimates were made at intervals over a period of some months so that the confidence limits incorporate the errors due to variations in all aspects of the tests.

The successful application of the analysis of Mather (1949) to the results is evidence that the distribution of surviving phage particles between the samples towards the end of the inactivating process is the same as that found amongst bacteria, the distribution being Poissonian. Interpretation of the results of phage inactivation tests in terms of the M.S.S.T. is therefore possible. Although the cycle of computations is too complex for routine use, the standard errors of the estimations have been calculated for the present results, and it has been found that the value of the M.S.S.T. estimated visually from the plot of contact time against log (—log proportion of negatives) falls within the calculated limits of error from both the first and second approximation. The reliability of this visual estimate is only acceptable when 10 or more replicate determinations have been performed using a contact time interval of 1/8 to 1/10 of the expected M.I.T. (or 1/6 to 1/7 of the M.S.S.T.).

Interpretation of extinction time data by means of the M.S.S.T. undoubtedly gives the most accurate interpretation of these data but it requires a high level of replication to have significance.

It has been shown that the relationship of the M.I.T. to the M.S.S.T. is constant over a wide range of extinction times. This is considered to indicate that at a high level of replication, the interpretation of the results in terms of the M.I.T. is as reliable as their interpretation in terms of the M.S.S.T. At a lower level of replication, estimates of the M.I.T. have been shown to have confidence limits of 10 to 20 per cent of the mean.

If these limits of accuracy are considered acceptable, the examination of the effect of chemical agents on phages may be carried out using 5 or 6 replicate determinations and the results expressed as the M.I.T. If a higher degree of reproducibility is required then a higher level of replication must be used and the results may then be best expressed as the M.S.S.T.

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THE *MITRAGYNA* SPECIES OF GHANA

THE ALKALOIDS OF THE LEAVES OF *Mitragyna stipulosa* (D.C.) O. KUNTZE

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The alkaloids rotundifoline, rhynchophylline, isorhynchophylline and isorotundifoline have been isolated from the leaves of *Mitragyna stipulosa* (D.C.), O. Kuntze (Rubiaceae). Traces of mitra-phylline were found in a few samples. The approximate concentration in various parts of the tree are recorded.

THE genus *Mitragyna* (Family—Rubiaceae) consists of about 10 species, all of which are trees growing exclusively in humid conditions. Three species occur in Ghana (Irvine, 1961) and also in other parts of West Africa (Aubreville, 1936; Dalziel, 1937). According to Hutchinson and other authorities at the Royal Botanical Gardens, Kew (1963), they are now designated: (i) *Mitragyna stipulosa* (D.C.) O. Kuntze; (ii) *Mitragyna ciliata*, Aubr. et Pellegr. and (iii) *Mitragyna inermis* (Willd.) O. Kuntze.

Various parts of all three species find use in local folk lore medicine for a wide variety of diseases: preparations of the plant are administered both orally and as local applications.

The presence of mitraphylline, rhynchophylline and rotundifoline have been reported; reports of previous investigations being summarised in Table I.

TABLE I

ALKALOIDS AND THEIR SOURCE REPORTED PREVIOUSLY

Source	Alkaloid	Reference
Mitragyna stipulosa (D.C.) O. Kuntze*		
Bark	unnamed	Michiels and Leroux (1925)
Bark	mitraphylline (rubradine)	Denis (1927)
Bark	mitraphylline	Michiels (1931), Raymond-Hamet and Millat (1935)
Bark	rhynchophylline	Larrieu (1930)
Bark	rhynchophylline	Raymond-Hamet and Millat (1934)
M. ciliata Aubr. et Pellegr.		
Bark	rhynchophylline	Ongley (1950)
Leaves	rotundifoline	Ongley (1950)
M. inermis (Willd.) O. Kuntze		
Bark	unnamed	Larrieu (1930)
Bark	rhynchophylline	Ongley (1950) Badger, Cook and Ongley (1950)
Leaves	mitrinermine†	Raymond-Hamet and Millat (1934) Millat (1946)

* No investigation of the leaf appears to have been made.
† Shown to be idential with rhynchophylline (Badger, Cook and Ongley, 1950).

* This work forms part of the thesis submitted by A. N. Tackie for the Ph.D. degree of the University of London.

Other alkaloids, reported from various species of *Mitragyna* grown elsewhere than West Africa, are summarised in Table II.

TABLE II
OTHER MITRAGYNA ALKALOIDS AND THEIR SOURCES

Alkaloid	Source	Reference
Mitraversine.. ..	.. *Mitragyna diversifolia* (leaves) Havil	Field (1921)
Mitragynine ..	.. *M. speciosa* (leaves) Korth	Field (1921) Ing and Raison (1939)
Mitraspecine ..	.. *M. speciosa* (bark, wood) Korth	Denis (1938)
Mitragynol	.. *M. rotundifolia* (Roxb.) O. Kuntze	Ongley (1950), Badger and others (1950)
Isorhynchophylline	.. *M. rubrostipulaceae* (Havil) (*Adina rubrostipulata* (leaves) Schuman)	Marion, Nair Edwards and Seaton (1960)

EXPERIMENTAL

Alumina used for column chromatography was Spence-type H, the adsorbent for thin layer chromatography was Alumina (Merck), and the solvent, chloroform.

All melting points are uncorrected. Equivalent weights were determined by non-aqueous titration (Beckett and Tinley, 1962). Elemental analyses were made by Mr. G. S. Crouch, School of Pharmacy, University of London and Drs. G. Weiler and F. B. Strauss, Oxford.

Materials. Flowering tops, leaves, stipules, young stem, old stem, stem bark and root bark were obtained from trees growing in the rain forest near the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, during various periods from January, 1961, to June, 1962. Details of the authentication of the species are reported by Shellard and Shadan (1963).

TABLE III
DISTRIBUTION OF ALKALOIDS IN *M. stipulosa*

Plant part	Rotundifoline	Rhynchophylline	Mixture of isorotundifoline and isorhynchophylline	Alkaloid $R_F \simeq 0.95$	Alkaloid $R_F \simeq 0$
Entire leaf	0·15	0·04	0·3	<0·001	0·001
Midrib	0·07	0·02	0·16	0	<0·001
Lamina	0·25	0·065	0·4	<0·001	<0·001
Stipules	0·3	0·15	0·4	0	0·003
Very young leaves *inside* stipules ..	0·8	0·13	1·12	0·045	0·005
Flowers	<0·001	0·005	0·005	0	0·003
Stem bark	0·002	0·02	0·05	0	0·001
Root bark	0	0	0	0	0
Node runner bark ..	0·01	0·006	0·03	0	<0·001

Preliminary Investigation and Distribution of Alkaloids

Various organs of *Mitragyna stipulosa* were examined for total alkaloidal content using a method based on that of Douglas and Kiang (1957). Dried leaves contained 0·3–0·45 per cent, the stem and root bark ~0·1 per cent and the flowering tops practically no alkaloid. The stipules contained ~0·85 per cent and the very young leaves inside the stipules

~2·0 per cent, but only very small quantities of these plant organs were available.

The alkaloids rotundifoline, isorotundifoline (hitherto known as mitragynol, Beckett and Tackie, 1963), rhynchophylline and isorhynchophylline were isolated from the leaves, some samples of which also furnished traces of mitraphylline (see later). In addition, traces of two other alkaloids were present.

By means of quantitative thin layer chromatography (details to be published elsewhere), the percentage of the different alkaloids in various parts of the plant was determined as reported in Table III.

Isolation of Alkaloids

Coarsely powdered leaves (1 kg.) were extracted by refluxing with 96 per cent ethanol (5 litres) and the extract evaporated under reduced pressure to a thin syrup. After acidifying with glacial acetic acid and diluting with a large volume of water, the precipitated non-alkaloidal matter was filtered off. This was dissolved in a little ethanol and treated with 5 per cent acetic acid, the acid washings being added to the filtrate which was made alkaline with ammonia and extracted with chloroform. The extract was washed, dried and evaporated to yield the crude alkaloids (4 g.). Bulked crude alkaloids (5 g.) were dissolved in benzene (10 ml.) and added to a column of powdered cellulose (Whatman No. 1, 30 × 2 cm.), the alkaloids being eluted with benzene (~1000 ml.) which on evaporation left a pale brown residue. Recrystallisation four times from absolute ethanol, gave rotundifoline (I) (1·5 g.) as colourless needles, m.p. 237–8° (Barger, Dyer and Sargent, 1939; Beckett and Tackie, 1963).

No further crystals could be obtained from the mother liquors and the solvent was removed. The residue (3·0 g.) was dissolved in benzene (10 ml.) and added to a column of alumina (30 × 2 cm.), the alkaloids being eluted with benzene (~1000 ml.) which on evaporation left a pale yellow residue. After decolourisation, recrystallisation from absolute ethanol: methyl ethyl ketone (2:1) yielded rhynchophylline (II) (0·2 g.), m.p. 212–214° (Nozoye, 1957; Seaton and Marion, 1957).

The mother liquors were evaporated to dryness and the residue (2·5 g.) dissolved in ether (10 ml.), the solution added to a column of alumina and the alkaloids eluted as under.

The following fractions were collected: (1) Ether (90 ml.), amorphous alkaloids. (2) Ether (150 ml.), amorphous alkaloids. (3) Ether-chloroform (1 : 1,100 ml.), rotundifoline (90 mg.). (4) Ether-chloroform (1 : 1,100 ml.), amorphous alkaloids. (5) Chloroform (40 ml.), amorphous alkaloids. (6) Chloroform (650 ml.), rhynchophylline (30 mg.).

Thin layer chromatography on alumina with chloroform as the running solvent was used to monitor all fractions collected in this analysis.

After removal of these crystallised alkaloids, the fractions were bulked, evaporated to give "uncrystallisable bases" (2·2 g.) which were dissolved in ether (10 ml.) added to an alumina column (30 × 2 cm.) and eluted with ether-chloroform (95 : 5); rotundifoline was found in the first few fractions, later fractions yielded a mixture of other alkaloids, while rhynchophylline remained on the column. After removal of the rotundifoline from the eluate the bulked fractions were evaporated to give an amorphous white residue (2·0 g.) which judged by thin layer chromatography, was free from rotundifoline and rhynchophylline. This residue in ether was extracted with 5 per cent sodium hydroxide and the resultant alkaline solution saturated with carbon dioxide to give a precipitate which was extracted with ether. Evaporation of the solvent left a residue which gave prismatic crystals from acetone-light petroleum (b.p. 40–60°) (1 : 1) (1·2 g.) m.p. 130–132°; prolonged drying under vacuum at 100° was needed to remove the acetone of crystallisation. This substance was identified as *isorotundifoline* (Beckett and Tackie, 1963).

III

The original ethereal solution from which the isorotundifoline had been removed by alkaline extraction, gave on evaporation, a white residue from which white needle crystals of isorhynchophylline (700 mg.) m.p. 144° (Seaton, Nair, Edwards and Marion, 1960) were obtained with some difficulty from n-hexane ether (1 : 1). Dried leaves (5 kg.) were collected in April, 1962, at the beginning of the rainy season, and a concentrated alcoholic extract prepared. After removal of rotundifoline, rhynchophylline, isorotundifoline and isorhynchophylline by the methods described above, the alkaline liquors still contained alkaloid which was only very sparingly soluble in ether. A chloroform extract, on evaporation, gave a residue which yielded white silky needles (0·24 g.) of mitraphylline (III), m.p. 267–268° (Seaton, Tondeur and Marion, 1958) from absolute ethanol. A further 0·11 g. of mitraphylline was obtained during the recrystallisation of the crude rotundifoline when a small fraction of this material, sparingly soluble in absolute ethanol, was recrystallised from large volumes of this solvent.

Characterisation of the Alkaloids

Rotundifoline. Soluble in acetone, chlorobenzene, chloroform, ethanol, sparingly soluble in ether, insoluble in sodium hydroxide, m.p. 239–40°. *Approximate R_F value, 0·54. $[\alpha]_D^{20}$ + 124·7° (*c*, 2 in $CHCl_3$); + 115·9 (*c*, 2 in EtOH). Found: C, 65·9; H, 6·9; N, 6·9; OMe, 15·3; equiv. wt., 400, 402. Calc. for $C_{22}H_{28}O_5N_2$†: C, 66·0; H, 7·0; N, 7·0; OMe, 15·5; equiv. wt., 400. pK_a 4·85 (electrometric titration in 80 per cent methyl cellosolve), 5·3 (electrometric titration in H_2O). λ_{max}(EtOH) 221 mμ (log ε 4·39), 289 mμ (log ε 3·42). Shoulder 242·5 mμ (log ε 4·14). λ_{min} 272 mμ (log ε 3·21). ν_{max} (Nujol) 3,240, 2,450 (broad) 1,700, 1,625, 1,275, 1,250, 1,107, 847, 780, 750, 730 cm.$^{-1}$. ν_{max} ($CHCl_3$) 3,440 cm.$^{-1}$.

The *perchlorate* crystallised from glacial acetic acid, m.p. 280–281°. Found: C, 52·9; H, 5·5; N, 5·6. $C_{22}H_{28}O_5N_2.HClO_4$ requires C, 52·7; H, 5·8; N, 5·6.

The *methiodide* crystallised from absolute methanol, m.p. 243·4°. Found: C, 51·5; H, 5·3; N, 5·2; equiv. wt. 540, 543. $C_{22}H_{28}O_5N_2$.MeI requires C, 50·9; H, 5·7; N, 5·2; equiv. wt., 542.

The *hydriodide* crystallised from ether-ethanol (1 : 1), m.p. 221–222°. Found: C, 50·1; H, 5·5; N, 5·1; equiv. wt., 530. $C_{22}H_{23}O_5N_2$.HI requires C, 49·3; H, 5·5; N, 5·3; equiv. wt., 528.

The *hydrobromide* crystallised from ether-ethanol (1 : 1), m.p. 215–217°. Found: C, 54·2; H, 6·4; N, 5·4; equiv. wt., 483. $C_{22}H_{28}O_5N_2$.HBr requires C, 54·9; H, 6·0; N, 5·8; equiv. wt., 481.

The *trinitrobenzene derivative* crystallised from absolute ethanol, m.p. 195–197°. Found: C, 54·2; H, 5·0; N, 11·5; OMe, 10·1; equiv. wt., 611·5; $C_{22}H_{28}O_5N_2.C_6H_3(NO_2)_3$ requires C, 54·8; H, 5·1; N, 11·4; OMe, 10·1; equiv. wt., 613.

This alkaloid is identical in melting-point, mixed melting-point, optical rotation, ultra-violet and infra-red spectra and R_F value (thin layer chromatography) with an authentic sample of rotundifoline from *Mitragyna rotundifolia* (Badger, Cook and Ongley, 1950) kindly supplied by Dr. J. D. Loudon.

Rhynchophylline. Soluble in acetone, chlorobenzene, chloroform, ethanol, slightly soluble in ether, insoluble in sodium hydroxide, m.p. 212–214°. Approximate R_F value, 0·15. $[\alpha]_D^{21}$ − 14·4° (c, 2, $CHCl_3$). Found: C, 68·7; H, 7·1; N, 7·4; OMe, 17·7; equiv. wt., 380. Calc. for $C_{22}H_{28}O_4N_2$, C, 68·8; H, 7·3; N, 7·3; OMe, 16·1; equiv. wt.. 384. pK_a 6·4 (electrometric titration in 80 per cent methyl cellosolve), 6·8 (electrometric titration in H_2O). λ_{max} (EtOH) 208·3 mμ (log ε 4·45), 243·3m μ (log ε 421), 282·0 mμ (log ε 2·93). λ_{min} 222·2 mμ (log ε 3·76), 277·8 mμ (log ε 2·89). ν_{max} (Nujol) 1,725, 1,700, 1,640, 1,280, 1,250, 1,180, 1,125, 1,100, 780, 745 cm^{-1}.

This alkaloid is identical in melting-point, mixed melting-point, optical rotation, ultra-violet and infra-red spectra and R_F value by thin layer

* Where R_F values are quoted, these refer to thin layer chromatography on alumina with chloroform as solvent.

† Amended by Beckett and Tackie (1963) from $C_{22}H_{26}O_5N_2$ (Barger, Dyer and Sargent, 1939; Ongley, 1950).

chromatography with an authentic sample of rhynchophylline from *Mitragyna rubrostipulaceae* (Seaton and Marion, 1957; Nozoye, 1958) kindly supplied by Dr. L. Marion.

Mitraphylline. Soluble in chloroform, sparingly soluble in acetone, ethanol, ether, insoluble in sodium hydroxide, m.p. 267–268°. Approximate R_F value 0·19. Found: C, 68·5; H, 6·6; N, 7·7; equiv. wt., 370, 372. Calc. for $C_{21}H_{24}O_4N_2$, C, 68·5; H, 6·6; N, 7·6, equiv. wt., 368. λ_{max} (EtOH) 208 mμ (log ϵ 4·67), 241·5 mμ (log ϵ 4·19), 281 mμ (log ϵ 3·03). λ_{min} 222 mμ (log ϵ 4·0), 274 mμ (log ϵ 2·99). ν_{max} (Nujol) 3,600, 3,250, 1,715, 1,700, 1,620, 1,290, 1,270, 1,190, 1,170, 1,105, 775, 760 cm.$^{-1}$. The *perchlorate* crystallised in silky needles, m.p. 235–237°. Found: C, 53·4; H, 5·3; calc. for $C_{21}H_{24}O_4N_2.HClO_4$, C, 53·7; H, 5·4.

This alkaloid is identical in melting-point, mixed melting-point, ultra-violet and infra-red spectra and R_F value (thin layer chromatography) with an authentic sample of mitraphylline from *Mitragyna rubrostipulaceae* (Seaton and Marion, 1957) kindly supplied by Dr. L. Marion.

Isorhynchophylline. Soluble in chloroform, ethanol, ether, insoluble in sodium hydroxide, m.p. 144°. Approximate R_F value 0·50. $[\alpha]_D^{20}$ + 8·6° (*c*, 2 in $CHCl_3$). pK_a 5·2 (electrometric titration in 80 per cent methyl cellusolve), 6·25 (electrometric titration in H_2O). λ_{max} (EtOH) 204 mμ (log ϵ 4·46), 239·8 (log ϵ 4·24). Shoulder 277·8 mμ (log ϵ 3·25). λ_{min} 220·8 mμ (log ϵ 4·02). ν_{max} (Nujol) 3,200, 1,695, 1,610, 1,600, 1,105, 750 cm.$^{-1}$.

The *perchlorate* colourless needles from ether-ethanol, m.p. 163–165°. Found: C, 52·8; H, 6·1; N, 5·0; OMe, 12·1. Calc. for $C_{22}H_{28}O_4N_2.HClO_4.H_2O$, C, 52·5; H, 6·2; N, 5·6; OMe, 12·3 per cent.

This alkaloid is identical in melting-point, mixed melting-point, optical rotation, ultra-violet and infra-red spectra and R_F value (thin layer chromatography) with an authentic sample of isorhynchophylline from *Mitragyna rubrostipulaceae* (Seaton, Nair Edwards and Marion, 1960) kindly supplied by Dr. L. Marion.

Isorotundifoline. Soluble in acetone, chloroform, ethanol, ether, sodium hydroxide, slightly soluble in water, insoluble in light petroleum, m.p. 130–132°. Approximate R_F value 0·44. $[\alpha]_D^{24}$ − 7·7° (*c*, 2 in $CHCl_3$. Found: C, 66·2; H, 6·9; N, 6·9; equiv. wt., 396, 398. $C_{22}H_{28}O_5N_2$ requires C, 66·0; H, 7·0; N, 7·0; equiv. wt., 400. pK_a 6·7 (electrometric titration in 80 per cent methyl cellusolve), 7·4 (electrometric titration in H_2O). λ_{max} (EtOH), 218 mμ (log ϵ 4·43), 289 mμ (log ϵ 3·49), shoulder 242 mμ (log ϵ 4·13), λ_{min} 270 mμ (log ϵ 3·31). λ_{max} (0·001N NaOH in 70 per cent EtOH), 238 mμ (log ϵ 4·38), 306·7 mμ (log ϵ 3·54), shoulder 222 mμ (log ϵ 4·26). λ_{min} 281·7 mμ (log ϵ 3·36). ν_{max} (Nujol) 3,250, 1,695, 1,685, 1,625, 1,605, 1,230, 1,140, 1,100, 1,095, 915, 900, 790, 770, 740 cm.$^{-1}$.

The *hydrochloride* crystallised in silky needles, m.p. 216°, from ether-ethanol (1 : 1). $[\alpha]_D^{25}$ − 40·8° (*c*, 2 in H_2O). Found: C, 58·9; H, 6·8; N, 6·0; $C_{22}H_{28}O_5N_2.HCl, H_2O$ requires C, 58·1; H, 6·7; N, 6·2.

This alkaloid was named *isorotundifoline* because it is identical in

melting-point, mixed melting-point, optical rotation, ultra-violet and infra-red spectra and R_F value (thin layer chromatography) with the isomeric base obtained by isomerisation of rotundifoline (Beckett and Tackie, 1963).

Other alkaloids. The presence of two other alkaloids of $R_F = 0$ and 0·95 was indicated by thin layer chromatography (alumina/chloroform).

Discussion

The isolation of five alkaloids from the leaves of *Mitragyna stipulosa* was facilitated by the use of thin layer chromatography as an aid to separation and identification. The presence of mitraphylline at the advent of the rainy season is an interesting biosynthetic problem and is being investigated further. Another interesting feature is the high alkaloidal content of the very immature leaves, inside the stipules, i.e. before they have been involved in the photosynthetic process.

The isolation of pure alkaloids from the plant permits pharmacological evaluation of chemical entities rather than crude screening of extracts of uncertain composition; pharmacological studies of these alkaloids are in progress.

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THE *MITRAGYNA* SPECIES OF GHANA

THE ALKALOIDS OF THE LEAVES OF *Mitragyna ciliata* Aubr. et Pellegr.

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Seven alkaloids, rotundifoline, isorotundifoline, rhynchophylline, isorhynchophylline, *ciliaphylline*, *rhynchociline* and *mitraciliatine* have been isolated from the leaves of *Mitragyna ciliata* Aubr. et Pellegr. The latter three are hitherto undescribed alkaloids.

IN 1950, Ongley examined the leaves of *Mitragyna ciliata* and isolated the single alkaloid rotundifoline (see also Badger, Cook and Ongley, 1950). Four and sometimes five alkaloids were obtained from the leaves of *M. stipulosa* with evidence of traces of others (Beckett, Shellard and Tackie, 1963). The two species are so closely related taxonomically (Aubreville, 1936) and so similar anatomically (Shellard and Shadan, 1963), that a further examination of the alkaloidal content of the leaves of *M. ciliata* has now been made to enable the alkaloidal content of the two leaves to be compared.

EXPERIMENTAL

Alumina used for column chromatography was Spence-type H, the adsorbent for thin layer chromatography was Alumina (Merck), and the solvent chloroform unless otherwise stated. Recorded R_F values are those obtained using this system. All melting-points are uncorrected. Equivalent weights were determined by non-aqueous titration (Beckett and Tinley, 1952). Elemental analyses were carried out by Mr. G. S. Crouch, School of Pharmacy, University of London and Drs. G. Weiler and F. B. Strauss, Oxford.

Materials

Leaves from trees growing in the savannah and fringing forest zone near Tarkwa in the Western Region of Ghana were collected during various periods from January, 1961, to June, 1962. Details of the authentication of the samples are given by Shellard and Shadan (1963).

Isolation of Alkaloids

An ethanolic extract prepared from 12 kg. of dried coarsely powdered leaves was treated as described by Beckett, Shellard and Tackie (1963), to obtain total crude alkaloid (37 g.). Thin layer chromatography showed at least four alkaloids to be present.

The crude alkaloids were dissolved in dilute sulphuric acid (250 ml.) and after extracting with ether the solution was made alkaline with

* This work forms part of a Ph.D. Thesis submitted by A. N. Tackie for the degree of Ph.D. of the University of London.

ammonia and re-extracted with ether. The ethereal extract was washed, dried and concentrated to yield prismatic crystals (12·9 g.) of alkaloidal material, m.p. 213–214° (material "A," subsequently shown to be a mixture of two alkaloids). Mixtures of Material "A" and rotundifoline were isolated from the mother liquors and were separated by adsorption and elution through alumina with 10 per cent chloroform in ether followed by chloroform to give rotundifoline (4·9 g.), m.p. 238°, and Material "A" (1·3 g.), m.p. 213° (see below).

The remaining mother liquor was exhaustively extracted with 5 per cent sodium hydroxide. The alkaline extract was acidified with sulphuric acid, made alkaline with ammonia and extracted with ether. Treatment of the ethereal extract by the method described in the previous paper (Beckett and others, 1963) gave colourless prisms of isorotundifoline (2·3 g.), m.p. 131°.

The ethereal mother liquors after this alkaline extraction were washed free from alkali with distilled water and evaporated to give a residue which was dissolved in chloroform and extracted with 5 per cent hydrochloric acid. The chloroform solution yielded a pale yellow residue which on recrystallisation from ether: light petroleum (b.p. 40–60°) (1 : 1), gave fine sandy crystals (1·3 g.), m.p. 141°, of a new alkaloid now named *mitraciliatine.*

The total acid extracts and washings were made alkaline with ammonia and extracted with ether: evaporation gave a residue (10·2 g.) which was shown by thin layer chromatography to contain two alkaloids which differed from those previously described. A solution of the perchlorates in chloroform was passed through a column of alumina (30 × 2 cm.) and the alkaloids eluted with 10 per cent chloroform in ether followed by chloroform. All fractions were monitored by thin layer chromatography. Later fractions contained a single substance isolated as fine colourless needles (5·5 g.), m.p. 178–180°. This is a new alkaloid now named *rhynchociline.*

Earlier fractions containing both alkaloids were evaporated to dryness and the residue, in ether, added to a column of alumina (30 × 2 cm.). Alkaloids were eluted with 1 per cent chloroform in ether. Earlier fractions contained a single substance, faster moving on thin layer chromatography than rhynchociline, and continual treatment in this manner of the bulked fractions containing both alkaloids resulted in the isolation of isorhynchophylline which crystallised with some difficulty from n-hexane-ether (1 : 1) in colourless needles (2·7 g.), m.p. 144°.

Examination of Material "A"

Thin layer chromatography using Kieselgel G and chloroform: acetone (5 : 4) showed Material "A" to be a mixture of two alkaloids. Material "A" (1·2 g.) was treated with 0·6 g. picric acid in methanol (6 ml.). Water was added to incipient crystallisation and after ~18 hr. a yellow picrate (600 mg.), m.p. 130–131° was obtained. This yielded a single alkaloid which recrystallised from acetone in colourless needles (110 mg.), m.p. 222–223°. This is a new alkaloid now named *ciliaphylline.*

The solution from which the ciliaphylline picrate was removed contained small quantities of an alkaloid which corresponds to rhynchophylline (thin layer chromatography using Kieselgel G and chloroform : acetone (5 : 4)).

Characterisation of the Alkaloids

Rotundifoline, isorotundifoline and isorhynchophylline were characterised by comparison of melting-points, mixed melting-points, infra-red spectra and approximate R_F values by thin layer chromatography with the corresponding alkaloids obtained from the leaves of *Mitragyna stipulosa*.

Ciliaphylline was soluble in acetone, chlorobenzene, chloroform, ethanol; slightly soluble in ether; insoluble in sodium hydroxide, m.p. 222–223°. Approx. R_F value 0·12. $[\alpha]_D^{25}$ $-89{\cdot}5°$ (c, 0·65 in $CHCl_3$). Found: C, 66·6; H, 7·7; N, 7·2; OMe, 21·3; equiv. wt., 390. $C_{23}H_{30}O_5N_2$ requires C, 66·8; H, 7·3; N, 6·8; OMe (3) 22·5; equiv. wt., 414. pK_a 6·75 (electrometric titration in 80 per cent methyl cellosolve), 7·5 (electrometric titration in H_2O). λ_{max} (EtOH) 222 mμ (log ϵ 4·44), 244 mμ (log ϵ 4·24), 287 mμ (log ϵ 3·46). λ_{min} 234 mμ (log ϵ 4·20), 276 mμ (log ϵ 3·40). ν_{max} (Nujol) 1,728, 1,705, 1,640, 1,620, 1,500, 1,380, 1,335, 1,300, 1,402, 1,180–1,300 (a number of fused bands), 1,150, 1,170, 780, 770, 740 cm.$^{-1}$.

The *picrate* crystallised in yellow prisms from aqueous methanol, m.p. 130–131° (decomp.). Found: C, 52·8; H, 5·1; N, 9·7. $C_{23}H_{30}O_5N_2$, $C_6H_3O_7N_3$, H_2O requires C, 52·7; H, 5·3; N, 10·6.

Rhynchociline was soluble in acetone, chloroform, ethanol, ether; insoluble in n-hexane, light petroleum, sodium hydroxide, m.p. 178–180°. Approx. R_F value 0·30. $[\alpha]_D^{22}$ $+6{\cdot}2$ (c, 2 in $CHCl_3$). Found: C, 66·6; H, 7·7; N, 7·3; equiv. wt., 387. $C_{23}H_{30}O_5N_2$ requires C, 66·8; H, 7·3; N, 6·8; equiv. wt., 414. $pK_a = 6{\cdot}7$ (electrometric titration in 80 per cent methyl cellosolve), 8·3 (electrometric titration in H_2O). λ_{max} (EtOH), 225 mμ (log ϵ 4·41), 242 mμ (log ϵ 4·24), 286 mμ (log ϵ 3·48). λ_{min} 238 mμ (log ϵ 4·22), 277 mμ (log ϵ 3·39). ν_{max} (Nujol) 3,525 (weak), 3,100, 1,685, 1,605, 1,270, 1,240, 970 (weak), 780, 730 cm.$^{-1}$.

The perchlorate crystallised in colourless needles from ethanol and water (1 : 1), m.p. 221–223°. Found: C, 53·5; H, 6·1; N, 5·6; OMe 17·7. $C_{23}H_{30}O_5N_2.HClO_4$ requires C, 53·6; H, 6·0; N, 5·4; OMe (3) 18·1.

Rhynchociline is identical with an isomer obtained by isomerisation of ciliaphylline in 50 per cent acetic acid or pyridine (unpublished observations).

Mitraciliatine. Soluble in acetone, chloroform, ethanol, ether; insoluble in light petroleum, sodium hydroxide, m.p. 140–141°. Approx. R_F value 0·32. Found: C, 68·8; H, 7·5; N, 6·83; OMe, 22·7; equiv. wt., 405. $C_{23}H_{30}O_4N_2$ requires C, 69·3; H, 7·6; N, 7·0; OMe, 23·4; equiv. wt., 398. λ_{max} (EtOH) 228 mμ (log ϵ 4·59), 292·4 mμ (log ϵ 3·91). Shoulders at 248·8 mμ (log ϵ 4·17), 284 mϵ (log ϵ 3·89). λ_{min} 289·5 mμ (log ϵ 3·84). ν_{max} (Nujol) 3,150, 1,690, 1,245, 1,105, 770, 730 cm.$^{-1}$.

The *perchlorate* crystalised in yellow prisms from ether-ethanol, m.p. 229–230°. Found: C, 55·3; H, 6·3; N, 5·6; OMe, 17·3. $C_{23}H_{30}O_4N_2$. $HClO_4$ requires C, 55·4; H, 6·2; N, 5·6; OMe (3) 18·7. With vanillin and hydrochloric acid it gives an intense blue colour.

Discussion

By using thin layer chromatography seven alkaloids have been identified from the leaves of *M. ciliata.* Four of these, rotundifoline, isorotundifoline, rhynchophylline and isorhynchophylline have been reported previously and also occur in the leaves of *M. stipulosa.* The hitherto unreported alkaloids *ciliaphylline* and *rhynchociline* are interconvertible by heating in acetic acid or in pyridine. These alkaloids are of the rhynchophylline pattern but possess an extra aromatic methoxy group.

The third new alkaloid, *mitraciliatine*, unlike the above is an indole alkaloid as indicated by its colour reaction and ultra-violet and infra-red spectra.

The presence of both oxindole and indole alkaloids in the same plant organs is unusual though it is not uncommon for them to occur in separate organs of the same plant. Mitragynine, which is known to be an indole (Field, 1921; Ing and Raison, 1939; Hendrickson, 1961; Joshi, Raymond-Hamet and Taylor, 1963), was isolated by Field (1921) from the leaves of *Mitragyna speciosa* while mitraspecine, an oxindole alkaloid (Raymond-Hamet, 1950), was isolated from the bark of this plant by Denis (1938). The significance of these results in relation to the biosynthesis of the mitragyna alkaloids is being investigated.

Acknowledgments. One of us (A. N. T.) thanks the Vice-Chancellor of the Kwame Nkrumah University of Science and Technology, Kumasi, for the award of a scholarship.

We are also grateful to Mr. Allman, Head of the Faculty of Pharmacy at the Kwame Nkrumah University of Science and Technology and to Josephine Armah and Andrew Afful for their valuable assistance in collecting the plant material and the preparation of some of the crude alkaloidal extracts.

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The two papers were presented together by Dr. Tackie.

DATURA SANGUINEA R. AND P., ITS STEM AND LEAVES

BY T. E. WALLIS AND MIRJANA KONJOVIC

From the Museum of the Pharmaceutical Society of Great Britain

Received May 1, 1963

Datura sanguinea R. and P., the Dark-red Datura, is a perennial species growing as a shrub or small tree in Peru and Ecuador. It has been grown in England and the chief alkaloid present is hyoscine. The structure of the leaves and stem is described and compared with other medicinal species of *Datura*. The characters by which the dried drug may be recognised and distinguished from stramonium are given. The pollen grains are described as an additional means of differentiation.

Datura sanguinea R. and P. is a Peruvian species of *Datura* and is widely used in Peru for medicinal purposes. Some years ago plants were grown in the open from seed at the Wellcome Chemical Works, Dartford and the aerial parts of the plants were examined chemically by Drey and Foster (1953) for their content of solanaceous alkaloids. The total alkaloidal content of the leaves varied from 0·34 to 0·51 per cent, the content of hyoscine being from 0·34 to 0·49 per cent. In view of these results the authors reported that "The aerial parts contain sufficient hyoscine to make the plant a possible commercial source of the alkaloid".

Plants of this species were also grown from the seed by Dr. J. M. Rowson, both in the greenhouse attached to the Museum of the Pharmaceutical Society of Great Britain and also at the Experimental Ground at Birdsgrove House, Derbyshire. The identity of the plants was confirmed by Dr. Rowson, who compared them with the descriptions given by Ruiz and Pavon (1799), by Humboldt, Bonpland and Kunth (1818), by Lindley (1834) under the name *Brugmansia sanguinea*, and by de Candolle (1852). Dr. Rowson also compared the plants with specimens at the Herbarium of the Royal Botanic Gardens, Kew, Surrey.

DESCRIPTION OF THE PLANT

The following is a description of the leaves and stem of *Datura sanguinea* R. and P.; the Dark Red Datura. *Habitat* Colombia, Ecuador and Peru at an altitude of 1840 metres (6000 ft.).

Habit

The plant is a perennial and may develop as an undershrub or a small tree with a hemispherical, leafy top and grows to a height of about 1·75 to 9 metres (6 to 30 ft.). The main stem is thick, terete, much branched above and has an ash-coloured bark; the principal branches are terete and fragile, pubescent and leafy especially in the younger, distal parts; dwarf shoots arise in the axils of the leaves of the main branches; see Fig. 1.

Leaves

The leaves are bright green. The *larger* leaves, growing on the main branches have an alternate phyllotaxis; *petiole* about 4 to 7 cm. long,

terete, channelled above and pubescent; *lamina* about 15 to 23 cm. long and 9 to 15 cm. broad, ovate with two to three broad triangular lobes on each side and shallow sinuses, softly pubescent on both surfaces; *venation* reticulate with 2 to 4 principal lateral veins on each side, ending in the apices of the lateral lobes; *apex*, acute to somewhat acuminate. Fig. 2A and Fig. 1. The *smaller* leaves of the dwarf shoots are in groups of three

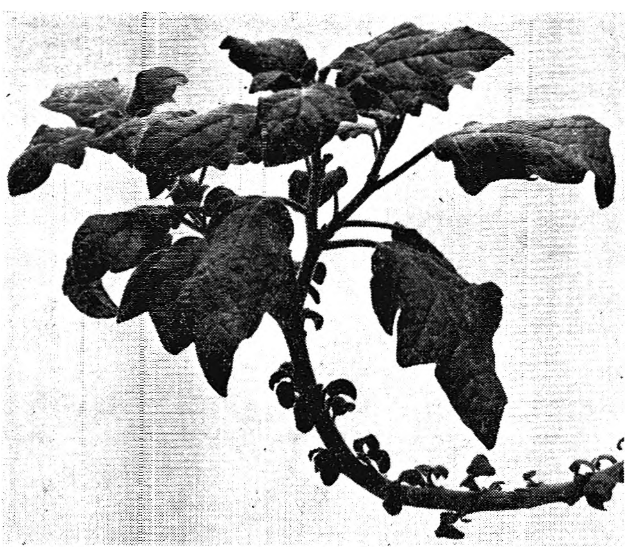

FIG. 1. *Datura sanguinea* R. and P. Branch of a shrub grown at Birdsgrove House, Derbyshire, showing the larger leaves and the dwarf, tufted shoots arising in their axils and bearing smaller leaves with entire margins. The larger principal leaves have fallen from the proximal portion of the stem shown in the photograph. 1/4 natural size.

to five; *petiole* about 0·5 to 2·0 cm. long; *lamina* about 5 to 12 cm. long and 1·5 to 4 cm. broad ovate, usually with an entire margin, occasionally somewhat wavy or having one or two broad triangular lobes; both lamina and petiole pubescent; *apex* acute; *venation* reticulate, lateral veins anastomosing near the margin; see Fig. 6A and Fig. 1.

HISTOLOGY OF THE LEAF

The leaves of *Datura sanguinea* R. and P. have a structure generally resembling that of the leaves of other important medicinal species of *Datura*, viz.: *D. stramonium* Linn., *D. tatula* Linn., *D. innoxia* Miller, *D. metel* Linn., as described by Timmerman (1927).

The larger leaves of the main branches, Fig. 2A, of *D. sanguinea* and the smaller leaves of the dwarf axillary shoots, Fig. 6A, have similar histological characters and the dimensions of the cells are slightly smaller for the smaller leaves (Fig. 5). The following description may be taken as applying to both the larger and the smaller leaves; unless otherwise stated, the measurements recorded refer to the larger leaves.

Petiole

The petiole of the larger leaves, measured at the middle point, is about 4 mm. thick; that of the smaller leaves of the dwarf shoots is about 1·5 to 2 mm. thick. A transverse section, Fig. 2B, is nearly circular in outline; on the upper side is a wide shallow groove, for the larger leaves about 1·5 mm. wide, flat or slightly convex at the bottom for a width of

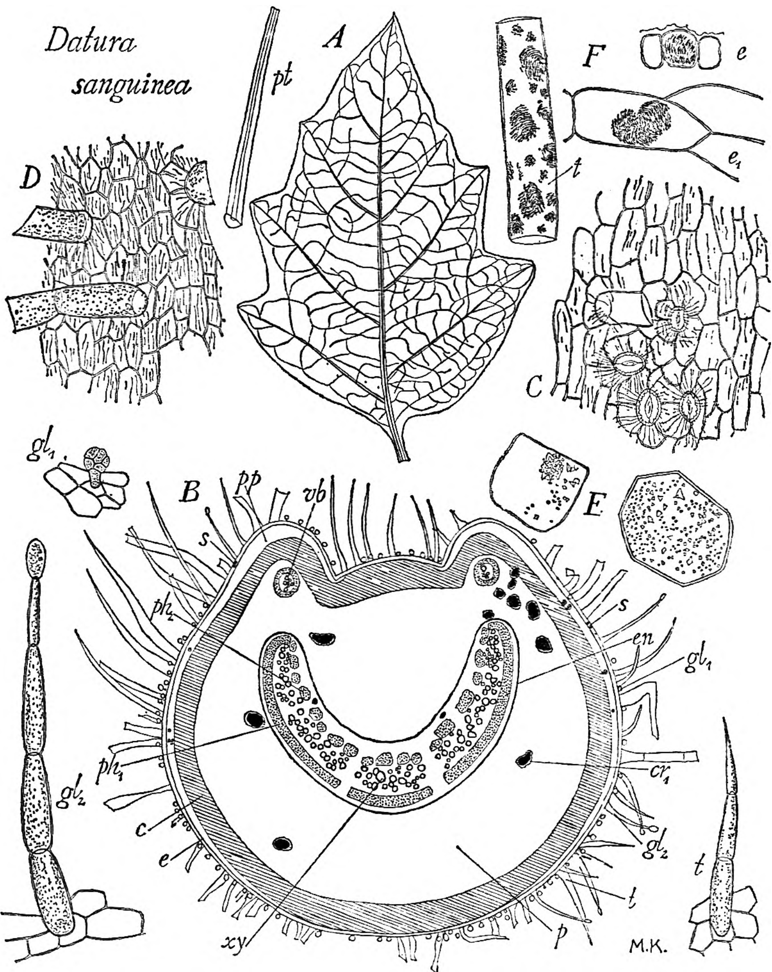

FIG. 2. *Datura sanguinea* R. and P. *A*, entire leaf, 1/3rd natural size, showing the undersurface and the separated petiole, *pt*, at one side. *B*, Petiole, T.S. × 16, diagrammatic. *C*, lower and *D* upper epidermis of the petiole, both × 70. *E*, cells from the parenchyma of the petiole, containing calcium oxalate crystals. *F*, Cells from the epidermis of the petiole, *e*, in T.S. and e_1, in surface view and a cell, *t* from a trichome, all showing groups of fine acicular crystals, × 130. *c*, collenchyma; cr_1, microsphenoidal calcium oxalate; *e*, epidermis; *en*, endodermis; gl_1, clavate glandular trichome; gl_2, glandular trichome with uniseriate stalk, × 70; p_1, parenchyma; ph_1, normal phloem; ph_2, perimedullary phloem; *p.p.*, photosynthetic parenchyma; *s*, stoma; *t*, covering trichome; *vb*, vascular bundle; *xy*, xylem.

1·0 mm., and having a rounded ridge, about 0·5 mm. high, on each side. In each of the ridges there is a small vasicentric vascular bundle. The meristele is gutter-shaped, about 2·25 mm. wide across the open side and 1·5 mm. deep; it is composed of a layer, about 0·5 mm. thick, of small vascular bundles, which have normal phloem on the abaxial surface and several small groups of perimedullary phloem on the adaxial surface, the whole group of bundles being surrounded by a starch sheath (endodermis) the cells of which contain a few small starch granules and some of them sphenoidal microcrystals of calcium oxalate. Cells filled with sphenoidal microcrystals also occur sparsely scattered throughout the cortical ground tissue of the petiole. The petiole is strengthened by a complete hypodermal layer of collenchyma, about 0·25 mm. wide. On the sides of the petiole, between the epidermis and the collenchyma and over the ridges there is a narrow layer of photosynthetic parenchyma. All parts of the epidermis bear numerous covering and glandular trichomes, Fig. 2, gl_1, gl_2, and *t*, which resemble the trichomes of the lamina. Some of the epidermal cells contain a group of fine acicular crystals, the identity of which has not been established; some of the trichomes contain similar crystals, Fig. 2, *F* and Fig. 6 cr_2. The cuticle of the epidermis is striated longitudinally, Fig. 2, *D* and *C*.

Lamina

At a position about one third of the length of the leaf from its base, the *midrib* projects strongly (about 2·2 mm. in the larger leaves) on the lower surface and is rounded in outline; over the midrib on the upper side there is a ridge about 0·5 mm. high; see Fig. 3, *A*. Both the projecting lower surface and the upper ridge are supported by three or four layers of hypodermal collenchyma. These and other features closely resemble the structure of the midrib of *Datura stramonium* Linn. There are a few scattered idioblasts with sphenoidal microcrystals in the fundamental parenchyma, Fig. 3, *A* cr_1, and the meristele resembles that of the petiole; the starch sheath, however, is not clearly developed on the upper, adaxial side.

Both the upper and the lower epidermises of the leaf bear very numerous conical covering trichomes 2 to **4** to **5** cells long and about 30 to **95** to 160 μ wide at the base, their total length is about 58 to **690** to 1536 μ; and the length of the basal cell is about 57 to **190** to 328 μ. Two kinds of glandular trichomes are present, a few clavate ones, Fig. 2, gl_1, with a one or two-celled stalk and a pluricellular pyriform head, similar to those of other solanaceous plants, and others with a long 2 to **4** to **5** to 6-celled uniseriate stalk and an ovoid or globular unicellular head, see Fig. 2, gl_2.

The *interneural lamina* is dorsiventral and resembles that of *D. stramonium* Linn. No stomata could be found on the upper epidermis, but they are very numerous in the lower, see Fig. 3, *C*, *D* and *E*. The palisade tissue consists of a single layer of cells and beneath it is a crystal layer, many of its cells each containing usually a cluster crystal of calcium oxalate, about 20 to 30 μ in diameter, occasionally a single prism is present in a cell. The anticlinal walls of the majority of the cells of the upper

epidermis are wavy and those of the lower epidermis very strongly wavy, see Fig. 3, *E* and *F*. The cells surrounding the bases of the trichomes have straight or nearly straight walls; those near the margin of the leaf also have straight walls, being rhomboid on the upper surface and elongated

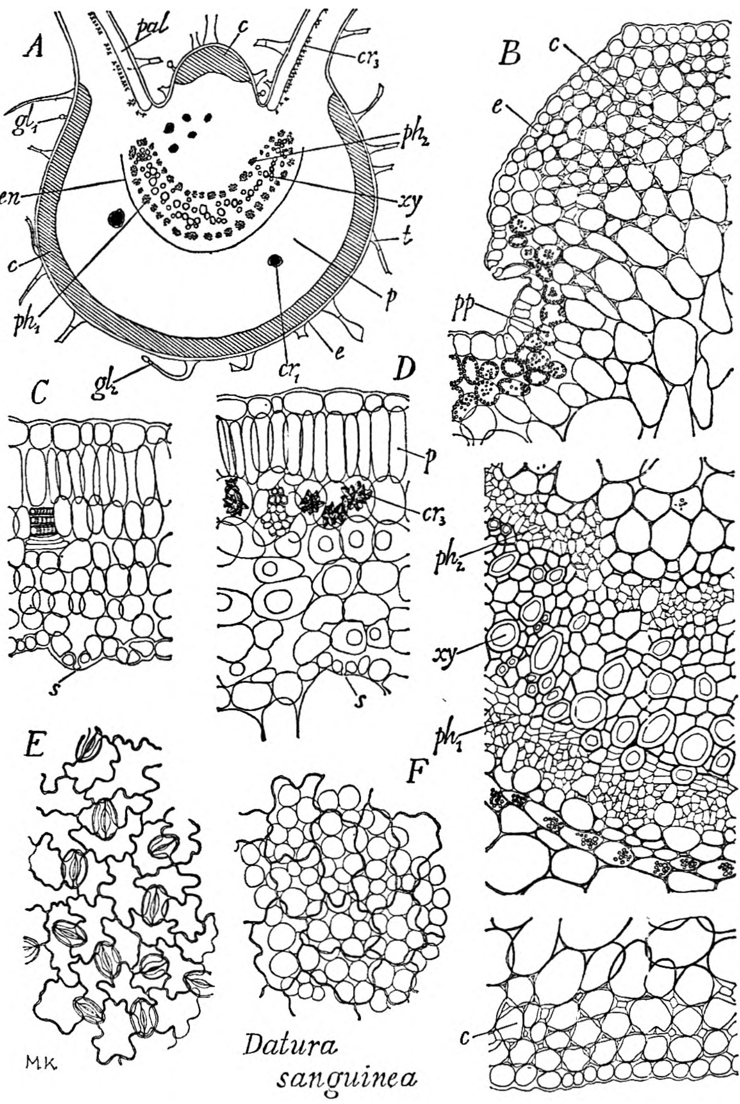

FIG. 3. *Datura sanguinea* R. and P. Lamina of larger leaf. *A*, diagrammatic T.S. of the midrib, × 16; *B*, details of the midrib, T.S., × 100; *C* and *D*, T.SS. of the lamina, *C* near the apex, *D*, at the middle, × 130. *E*, lower epidermis, × 130; *F*, upper epidermis and palisade, × 130. *c*, collenchyma; cr_1, microsphenoidal calcium oxalate; cr_3, cluster crystals of calcium oxalate; *e*, epidermis; *en*, endodermis; gl_1, clavate glandular trichome; gl_2, uniseriate glandular trichome; *p*, parenchyma; *pal*, palisade; ph_1 normal, phloem; ph_2, perimedullary phloem; *pp*, photosynthetic parenchyma; *s*, stoma; *t*, covering trichome; *xy*, xylem.

on the lower surface. The *stomata* are sometimes anisocytic (cruciferous) and vary somewhat in size in the apical quarter of the leaf from 27 by 20 μ to 42 by 31 μ; in the central region from 40 by 27 μ to 70 by 40 μ; near the margin and in the basal region from 23 by 20 μ to 42 by 27 μ. The *palisade ratio* varies from 5 to 7 to 8 and the stomatal index for the lower surface from 22 to **25** to 30. The vein-islets are very similar in shape and size to those of *D. stramonium*; the bounding lines tend to be somewhat more curved and the islets slightly larger, being on the average about 8 per sq. mm. against 11 for *D. stramonium.* They are of little use for differentiation, see Fig. 4.

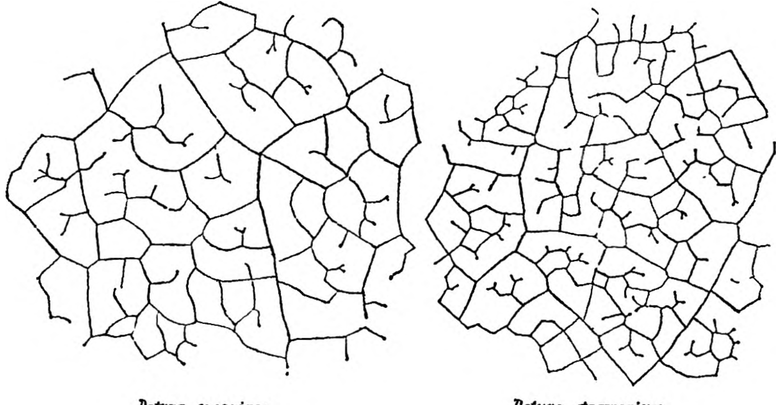

FIG. 4. *Datura sanguinea* R. and P. and *Datura stramonium* Linn., vein-islets, × **14.**

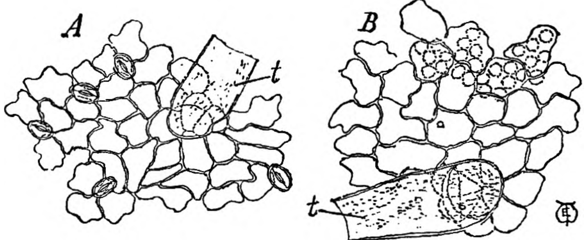

FIG. 5. *Datura sanguinea* R. and P. Smaller leaf. *A*, lower epidermis; *B*, upper epidermis; *t*, base of trichome, × 130.

HISTOLOGY OF THE STEM

The structure of the stem shows a general resemblance to that of *D. stramonium* Linn. (Timmerman 1927); the cells of the epidermis are about 40 to 75 μ in diameter and occasional ones contain sphenoidal micro-crystals of calcium oxalate, while others may sometimes contain groups of small unidentified acicular crystals similar to those found in the epidermis of the petiole, see Fig. 6, *D*. The young stem, Fig. 6, *B* and *C*, up to a diameter of about 1·0 cm., is densely covered with uniseriate glandular trichomes consisting of 4 to 5 to 8 cells and up to 2 mm. long; their basal

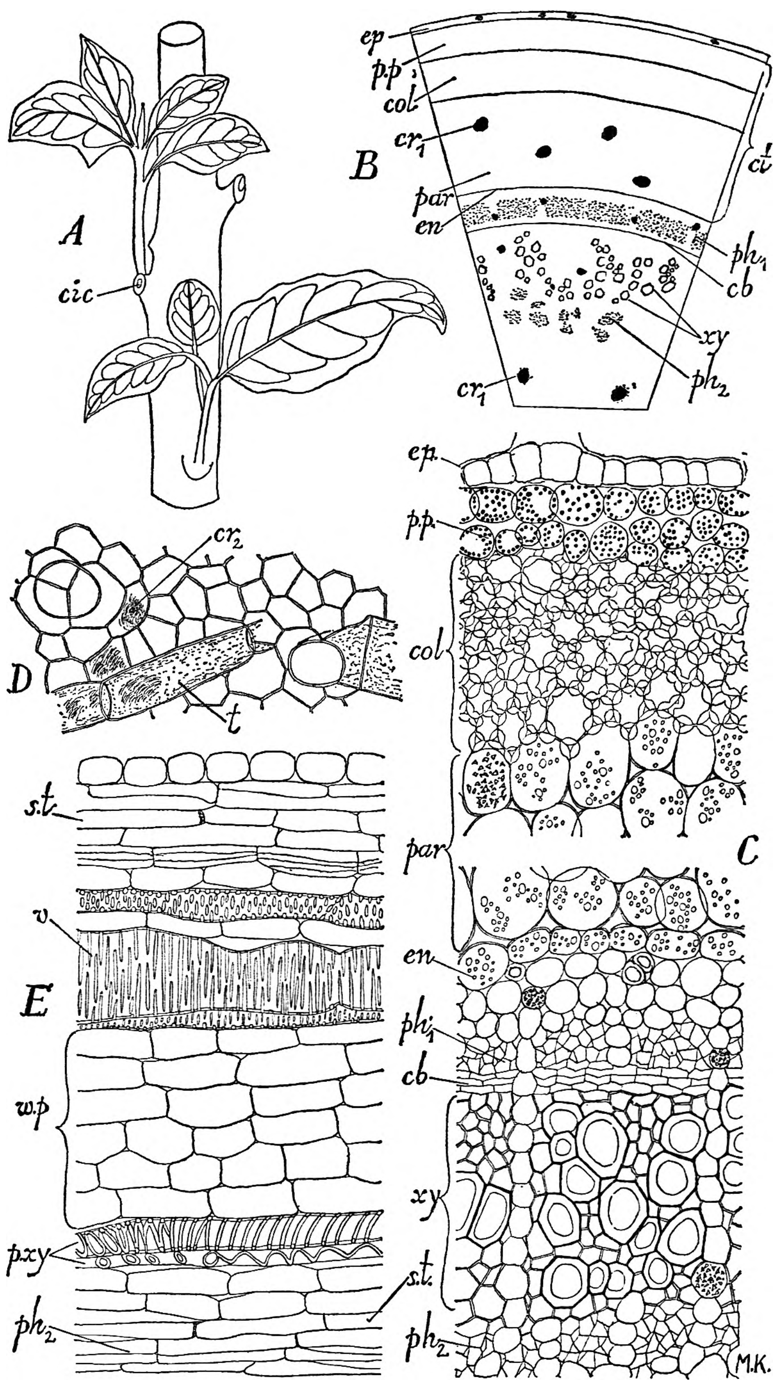

FIG. 6. *Datura sanguinea* R. and P. Stem. *A*, habit sketch showing scars, *cic*. of the larger leaves and two dwarf axillary shoots with small leaves, × 1/3; *B*, diagrammatic T.S. × 16; *C*, details of T.S. × 70; *D*, epidermis in surface view, × 70; *E*, L.S. through the outer part of the stele, × 70; *cb*, cambium; *cic*, cicatrix; *col*, collenchyma; cr_1, microsphenoidal calcium oxalate; cr_2, fine acicular crystals; *ct*, cortex; *ep*, epidermis; *en*, endodermis; *par*, parenchyma; ph_1, normal phloem; ph_2, perimedullary phloem; *p.p.*, photosynthetic parenchyma; *p.xy*, primary xylem; *s.t.*, sieve tissue; *t*, trichome; *v*. vessel; *w.p*, xylem parenchyma; *xy*, *xylem*.

cell is slightly tapered towards the base, which is about 70 to 90 μ wide and midway up the diameter is up to 150 μ; the glandular unicellular head is about 35 to 40 μ in diameter. Non-glandular trichomes appear to be absent from the stem. The older stems are covered externally with a layer of cork derived from a phellogen which arises in the epidermis, Fig. 7, *A*. Beneath the epidermis are two to four rows of photosynthetic parenchyma containing chlorophyll granules; within this is a ring of collenchyma about 0·3 mm. wide consisting of 6 or 7 rows of cells; the remainder of the cortex, about 0·5 mm. wide, consists of round-celled, thin-walled parenchyma containing small starch granules about 3·5 to 7·5 μ in diameter, the cells being about 95 to 155 μ in diameter, a few scattered cells are filled with sphenoidal microcrystals of calcium oxalate.

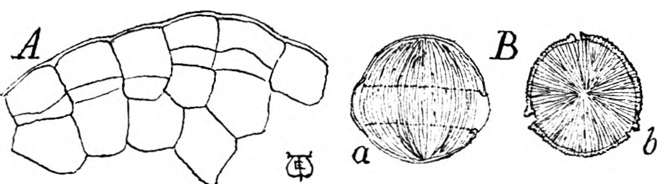

FIG. 7. *Datura sanguinea* R. and P. *A*, Origin of phellogen in the epidermis of the stem × 180; *B*, pollen grains; *a*, equatorial view; *b*, polar view × 300.

The endodermis is formed of rather smaller cells, about 60 to 80 μ in diameter, containing starch granules, Fig. 6, *C*. The pericycle consists of parenchyma in which, immediately within the endodermis, at intervals, there are fibres either singly or in groups of 2 to 4, they are about 33 to **48** to 55 μ in diameter. These fibres are unlignified and are very evident in the older stem, but are not so clearly visible in the younger parts of the stem. The phloem forms a band about 0·3 mm. wide. The xylem is a region about 0·5 mm. wide; it is rather parenchymatous and is traversed by medullary rays. On the inner side of the xylem, in the periphery of the pith, are numerous groups of perimedullary phloem. The pith consists of large-celled parenchyma with scattered idioblasts filled with sphenoidal microcrystals of calcium oxalate; similar idioblasts occur in the parenchyma of the medullary rays and of the xylem and phloem. The cells of the medullary rays and pith, other than the idioblasts, contain small starch granules similar to those in the cortex.

POLLEN GRAINS

Leaves, especially densely pubescent ones, collected from plants which are in flower, as is usual for stramonium, will often have pollen grains adhering to the surface. The characters of the pollen are therefore a useful additional means of identification. The pollen grains of *D. sanguinea* differ markedly from those of *D. stramonium* and are therefore described as affording a useful diagnostic character; they do, however, closely resemble the pollen of *D. innoxia* (Wallis and Rohatgi, 1952).

The pollen grains of *Datura sanguinea* are sub-spherical having a polar diameter slightly smaller than the equatorial diameter. When examined

TABLE I

MEDICINAL SPECIES OF *DATURA*

	D. stramonium	*D. tatula*	*D. innoxia*	*D. metel*	*D. sanguinea*
Covering trichomes					Few;
Length	130 to 275 to 550 µ	130 to 390 to 650 µ	120 to 325 to 450 µ	About 200 to 500 µ	58 to 690 to 1536 µ
Width at base	35 to 61 to 95 µ	50 to 110 µ	35 to 50 µ	10 to 30 µ less than 35 µ	38 to 94 to 160 µ
Length of basal cell	75 up to 120 µ or more	75 up to 240 µ or more	Often over 50 µ	50 µ rarely up to 75 µ	57 to 190 to 328 µ
Number of cells	1 to 3 to 4	3 to 4 to 8	1 to 3 to 4	1 to 6	2 to 4 to 5 to 9
Stomatal index					
Upper epidermis	16 to 18 to 20	16 to 20 to 22	12·7 to 17·5 to 20	About 17	0
Lower epidermis	24 to 25 to 26	28 to 30 to 31	21 to 22 to 24	About 21	22 to 25 to 30
Palisade ratio	4 to 7 not less than 4	4 to 6 approx.	4 to 5 approx.	4 to 5 approx.	5 to 7 to 8
Glandular trichomes	Clavate, 1 to 2-celled stalk 30 to 50 µ long by 12 µ wide. 2 to 7 celled pyriform head (40 to 47 µ) × 45 µ	As *D. stramonium*	Many 2 to 4-celled, uniseriate, 75 to 600 µ long, with a unicellular rounded head. A few clavate similar to *D. stramonium.*	Similar to *D. stramonium*	Many clavate similar to *D. stramonium.* Also many with 2 to 5 to 8-celled uniseriate stalk up to 800 µ or sometimes 2 mm. long, with a unicellular glandular head.
Pollen grains	Sub-spherical 56 to 80 µ diameter. 3 pores 24 to 28 µ; very slight furrows; exine irregularly warty.	As *D. stramonium*	Sub-spherical 69 to 84 µ diameter. 3 pores 12 to 24 µ; small germinal furrows, exine with striae radiating from the poles.	No record	Sub-spherical 45 to 54 µ diameter. 3 pores 8 to 16 µ; no furrows visible; exine with striae radiating from the poles, an equatorial band about 15 µ wide.

in a 70 per cent w/v solution of chloral hydrate in water, the polar diameter measures about 45 to 47 μ and the equatorial diameter measures about 50 to 54 μ; when mounted in lactophenol the dimensions are slightly smaller. The surface of the exine is striated with slight ridges radiating from the poles to the equator where about 100 can be counted. There are three pores, but no germinal furrows are visible. When viewed equatorially many grains show a faintly outlined band, about 15 μ wide, passing round the equator, see Fig. 7, *B*.

Conclusion

The structure of the leaves of *D. sanguinea* closely resembles that of *D. stramonium* and other medicinal species of *Datura*. The most distinguishing features are:

(1) The abundance of trichomes upon, and the absence of stomata from, the upper epidermis.

(2) The dimensions of the basal cell of the trichomes, which is about 57 to **190** to 328 μ long and 38 to **94** to 160 μ wide at the base.

(3) The abundant uniseriate glandular trichomes up to about 0·8 to 2·0 mm. long, with a unicellular rounded head.

(4) Pollen grains which differ markedly from those of *D. stramonium*, but have much resemblance to those of *D. innoxia*.

Note. In a powder of the leaves, trichomes are much broken hence their length and the number of cells are of little value as characters of the powdered leaf.

Table I summarises the important characters of five well-known medicinal species of *Datura*.

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The paper was presented by Dr. Wallis.

Short Communication

A DEPENDENCE ON WATER CONTENT OF BACTERICIDAL EFFICIENCY OF GAMMA-RADIATION

By A. Tallentire, N. A. Dickinson and J. H. Collett
From the Department of Pharmacy, University of Manchester

Received May 1, 1963

The bactericidal efficiency of ionising radiations on cells irradiated at a controlled water content is governed by the nature of the environment during and after irradiation. Furthermore, recent experiments with soft X-rays show that for given conditions of irradiation and post-treatment, lethal efficiency can depend on water content (Tallentire and Powers, 1963). Quantitative data describing these relationships for radiations of greater energies are important on two accounts: (1) to allow recognition of physical and/or chemical processes occurring soon after energy absorption in irradiated cells, and (2) to provide a more complete understanding of the microbial aspects of "radiation sterilisation".

Spores of *Bacillus megaterium* (ATCC 8245) distributed on kaolin (Tallentire and Davies, 1961) were used for the experiments. After secondary drying (Tallentire and Dickinson, 1962) weighed samples were equilibrated in vacuum to known H_2O vapour pressures. Gamma-irradiation (cobalt-60) of samples of spores of different water content was carried out at 22° in the absence of oxygen. All samples were given an anoxic post-irradiation soaking with liquid H_2O before exposure to the atmosphere; this procedure avoids lethal damage from the post-irradiation oxygen effect. Surviving fractions, based on colony counts, give exponential dose/survival curves and the slopes of these are used as a measure of lethal efficiency of the radiation, higher values for the slopes indicating greater efficiency.

The range of equilibrium H_2O vapour pressures tested was from 5×10^{-4} to 21 torr. Fig. 1 shows that over this range the limiting values of the slopes are 7·7 and 10·4 $Krad^{-1} \times 10^3$. Thus, in changing from a condition in which the spores are driest to one in which they are in equilibrium with H_2O vapour at a partial pressure equivalent to 100 per cent relative humidity at room temperature (23°), the lethal efficiency of the gamma-radiation increases by about 35 per cent. The transition from low values of slopes to high values occurs over that section of the vapour pressure range between 4·6 and 8·0 torr. Below 4·6 torr, radiation efficiency is unaffected by a ten thousandfold decrease in H_2O vapour pressure and above 8·0 torr, when efficiency is highest, it remains unchanged over a threefold increase in equilibrium pressure. It is of particular interest that the same high efficiency is also seen when spores and kaolin are soaked in liquid H_2O during irradiation, a condition in which the spores can be expected to be fully hydrated. Using 50 Kvap X-rays, Tallentire and Powers (1963) have irradiated spores of the same strain of *B. megaterium* mounted on cellulose acetate discs and found a similar

relationship. Here a 25 per cent increase in efficiency was observed on increasing H_2O pressure from 5×10^{-2} to 22 torr. The present experiments using gamma-rays and an insoluble inorganic substrate show that the increase (at 4·6 to 8·0 torr) occurs between the two wider pressures tested with X-rays and in addition, that the critical section of the vapour pressure range is narrow in comparison with those sections over which efficiency is invariant.

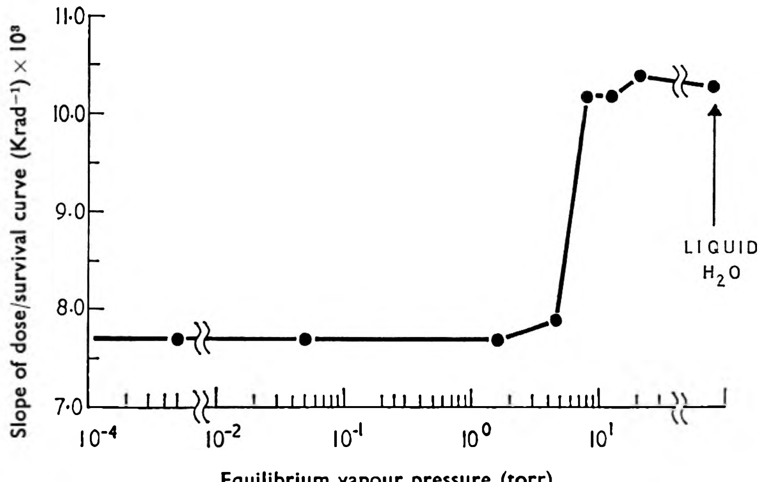

FIG. 1. Slopes of exponential dose/survival curves from anoxic gamma-irradiation of *B. megaterium* spores equilibrated to different H_2O vapour pressures.

We are not yet in a position to identify mechanisms by which changes in water content in our system alter radiation efficiency, but we have clearly established that a part of the lethal damage induced in spores by gamma-radiation can be mediated by water. Moreover, our findings are important from the viewpoint of "radiation sterilisation". We note that H_2O vapour partial pressures commonly encountered in working atmospheres may lie on either side of the critical section mentioned above and in this context our results further stress the need for consideration of known factors, which affect bactericidal efficiency, when selecting a working radiation dose.

Acknowledgement. This work was carried out under U.K.A.E.A. agreement No. EMR/1549.

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The paper was presented by DR. TALLENTIRE.

A COMMON ANTIGEN IN THE CELL WALLS OF THREE LYSOZYME-SENSITIVE BACTERIA

By David Wiseman

From the Department of Pharmacy, Bradford Institute of Technology

Received April 29, 1963

Isolated cell walls from three lysozyme-sensitive bacterial species, *Bacillus megaterium*, *Micrococcus lysodeikticus* and *Sarcina lutea*, have been shown to contain a common antigen. This antigen has been demonstrated by agglutination and agar precipitation techniques.

Several workers have demonstrated the presence of specific antigens in the isolated cell walls of bacteria (Cummins, 1954; McCarty, 1952; Salton, 1952, 1953; Tomesik and Guex-Holzer, 1954; Vennes and Gerhardt, 1956). Such isolated cell walls from some Gram-positive bacteria are susceptible to lysis by lysozyme and it was felt that these walls might contain components which were both common substrates for lysozyme and also common antigens. The following work was carried out to investigate this possibility.

Methods and Materials

Crystallised egg white lysozyme was obtained from the Armour Pharmaceutical Co. Ltd., Eastbourne, England.

Organisms: Bacillus megaterium strain K.M. (non-sporing strain); *Micrococcus lysodeikticus* N.C.T.C. 2665; *Sarcina lutea*, N.C T.C. 8340.

Cultures were grown in Difco nutrient broth in 2 litre flasks with forced aeration at 30° for 18 hr. The cells were harvested by centrifuging washed twice with distilled water and resuspended in M/15 phosphate buffer.

Cell wall preparation. Cell walls were prepared by the method of Salton and Horne (1951). Cell suspensions containing 10 mg./ml. dry wt. were mixed with Ballotini and shaken in a Mickle disintegrator for 10 min. The ruptured cell walls were collected by centrifuging. washed twice with distilled water, treated with trypsin for 30 min. and finally washed once in M sodium chloride then twice with distilled water. The washed cell walls were resuspended in M/15 phosphate buffer containing 0·001 per cent thiomersal as a preservative and were stored at 4°.

Lysozyme digests. Lysozyme digests of cells and of isolated cell walls were prepared by treating suspensions of cells (20 mg./ml.) or of walls (1 mg./ml.) in buffer with 100 μg. of lysozyme per ml. at 37° for 30 min. The resulting solutions were centrifuged to remove any residual particles and were stored at 4°: they were used within 72 hr. of preparation.

Antisera. Antisera against intact cells, isolated cell walls and lysozyme digests of cell walls from all three organisms were prepared in rabbits. The equivalent of 1 mg. dry wt. of cell walls was injected intravenously into rabbits every 2 days for a total of 22 days. Three weeks after the last injection 20 ml. of blood was removed from the ear vein of each rabbit, and the serum separated from the blood cells by centrifuging. 0·001 per

cent thiomersal was added as a preservative and the sera stored at 4° until required.

Agglutination titres. Agglutination titres of the antisera were measured against their homologous and heterologous cells and cell walls by mixing equal volumes of cell or wall suspensions (1 mg./ml.) and of diluted antisera. These mixtures were incubated in agglutination tubes at 37° for 24 hr. and examined for agglutination.

Agar diffusion precipitation reactions. Agar diffusion precipitation reactions between the antisera and the digests of intact cells and cell walls were made in a 1 per cent gel of washed agar in M/15 phosphate buffer at pH 7·2 containing 0·001 per cent thiomersal. 20 ml. volumes of the agar gel were poured into pyrex petri dishes and reservoirs cut in the agar with a 6 mm. cork borer. One reservoir was placed in the centre of the dish and was surrounded by five further reservoirs evenly spaced with their inner edges 10 mm. from the edge of the central one.

One antiserum was placed in the central hole in each plate and the digests in the surrounding reservoirs. The plates were sealed with plasticine and incubated at 37° in an airtight perspex box and examined at 24 hr. intervals for the development of precipitation bands.

RESULTS

Using the injection schedule described above antibody titres reached a maximum level three weeks after the last injection but then fell off rapidly unless maintained by booster injections.

The results of agglutination tests are shown in Table I.

TABLE I

Antisera	Antigens					
	K.M.c.	K.M.w.	M.L.c.	M.L.w.	S.L.c.	S.L.w.
K.M.c.	512	128	16	16	8	8
K.M.w.	128	128	16	16	4	8
M.L.c.	8	8	256	64	16	16
M.L.w.	8	8	128	128	32	64
S.L.c.	2	4	8	8	128	16
S.L.w.	4	8	16	16	32	32

Figures are the reciprocals of agglutination titres.
K.M. = *Bacillus megaterium.* M.L. = *Micrococcus lysodeikticus.* S.L. = *Sarcina lutea.* c. = intact cells. w. = isolated cell walls.

These results show the presence of antigens specific to each organism but also an antigen or antigens common to all three species. The intact cells also show the presence of some surface antigens which are lost during the preparation of cell walls by the method described above.

Agar precipitation reactions give three or four bands of precipitation between each antiserum and its homologous digests. Two of these precipitation bands are still obtained when the antisera are tested against the heterologous digests. One of these common bands is however obtained when the reaction is carried out between the anti-digest sera and a solution of lysozyme and is thus due to the antigenic properties of the lysozyme. The remaining common band cannot however be accounted

for in this way and is also obtained when the digests are tested against antisera prepared against undigested cells or cell walls. These results indicate that there is at least one antigenic component which is common to the walls of all three species of bacteria.

Further work by the author (unpublished) has shown that this common component is present in the non-dialysable fraction of the cell wall lysozyme digests and is also still present when the walls are digested with the F Enzyme isolated from *Streptomyces albus* by Ghuysen (1957).

Discussion

It has been suggested that the immunological specificity of bacterial cells is carried in part by the mucopeptides in the cell walls (Rogers 1962). These mucopeptides occur in large amounts in the cell walls of Gram-positive bacteria particularly in those of lysozyme-sensitive organisms where they can account for up to 90 per cent of the dry weight of the walls. Such mucopeptides probably consist of polysacharide chains made up of *N*-acetyl glucosamine and *N*-acetyl muramic acid linked together at points by peptide chains of three or four amino-acids. Such molecules possess the physical properties required of antigens and variation in the composition and position of the peptide chains would be expected to give antigens of different specificity.

It seems probably from the above results that organisms which are highly sensitive to lysozyme have at least one common mucopeptide in their cell walls. Further work with other bacterial species is expected to confirm this.

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THE ACTION OF TETRACYCLINE AND CHLORAMPHENICOL ALONE AND IN ADMIXTURE ON THE GROWTH OF *ESCHERICHIA COLI**

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The dependence of *E. coli* growth rates upon chloramphenicol concentration has been established. Chloramphenicol and tetracycline are equivalent in their action on *E. coli* and their combined effects on growth rate are additive. The decrease in rate of growth is a function of the first power of concentration of either antibiotic or combinations of them. A new mechanism of action is implied when the growth rate decreases to negative values: at this stage the rate of decrease is a function of a fractional power of the concentration.

IN a previous paper the dependence of *Escherichia coli* growth rates upon tetracycline concentration and also the reproducibility of the methods was demonstrated (Brown and Garrett, 1963). It was found that where there was a positive but decreased growth rate in the presence of tetracycline the inhibitory effect was a function of the first power of the tetracycline concentration. It is intended to set up kinetic dependencies for separate antibiotics and to predict from these the rate of change of viables in the presence of combinations of them. A theoretical basis for this approach, including classification and definitions of combined antibiotic activity has been published previously (Garrett, 1958).

Using turbidimetric techniques based on the principles previously defined by Elion, Singer and Hitchings (1954), Ciak and Hahn (1958), showed that the action of chloramphenicol and tetracycline on the growth of *E. coli* was additive in the sense that equipotent units of these antibiotics when added to each other gave the same activity as an activity-equivalent amount of each antibiotic alone. These antibiotics are thus convenient to use in an initial venture into the prediction of combined antibiotic action, since it would seem possible on the basis of separate growth rate studies to establish an equipotency factor.

The purpose of this paper is to present results of investigations to ascertain the validity of these *à priori* predictions.

METHODS

E. coli strain E/r was the test organism and replicate slopes were used for each experiment. Details of experimental procedures have been described previously (Brown and Garrett, 1963). Bacto Antibiotic Medium 3, buffered at pH 7 was used for broth cultures and plate counts.

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Assayed samples of tetracycline hydrochloride (995 μg./mg.) were supplied by courtesy of the Upjohn Company and chloramphenicol by courtesy of Parke, Davis and Company. Antibiotic solutions were sterilised by filtration and replicate samples stored frozen. Fresh samples were used for each experiment.

Growth Rate Measurements

An overnight broth culture was diluted into broth at 37·5° and the growth rate followed by means of a Klett-Summerson colorimeter. At a predetermined optical density, such that the bacterial cells were known to be in the logarithmic phase, samples were further diluted into the required number of replicate volumes of broth maintained at 37·5°. This procedure reduces any lag phase to a minimum (Brown and Garrett, 1963). Dilutions of the replicate cultures were then plated out at intervals.

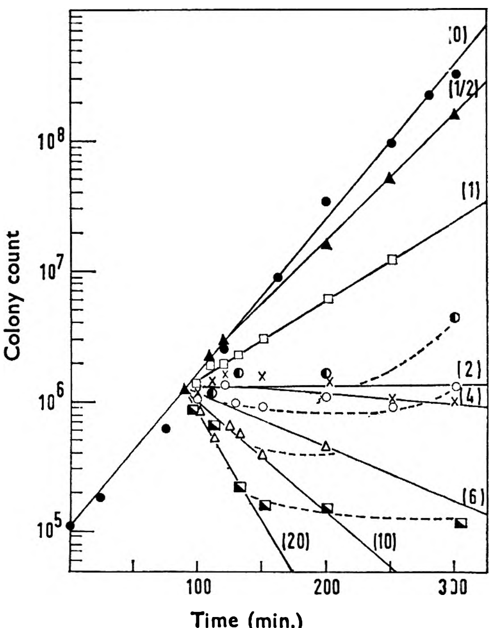

Fig. 1. Growth curves for *E. coli* in the presence of graded concentrations of chloramphenicol (μg./ml.).

Effect of Antibiotic Concentration

Eight replicate cultures of logarithmic phase cells were allowed to grow at 37·5° and after a predetermined interval, seven were inoculated with chloramphenicol solution. The cultures then contained 20, 10, 6, 4, 2, 1, 0·5 and zero μg./ml. of antibiotic. Colony counts were made at intervals and the results are illustrated in Fig. 1. Similar experiments for tetracycline have already been reported in greater detail (Brown and Garrett, 1963).

The values of the slopes (S_i), for the initial straight line part of the curves for the plot of logarithm colony count against time were determined: this represents a pseudo first order rate constant for *E. coli* growth. The error of estimate in these cases was of the order of 10 per cent for the data (Fig. 1) obtained within an hour after the addition of the antibiotic to the microorganisms in the logarithmic growth phase. The variation was greatest in those instances where a net increase of viables was not observed. Each value of S_i was subtracted from the value of the slope in the absence of any antibiotic (S_o). Logarithm ($S_o - S_i$) was plotted against logarithm antibiotic concentration for both tetracycline and chloramphenicol (Fig. 2).

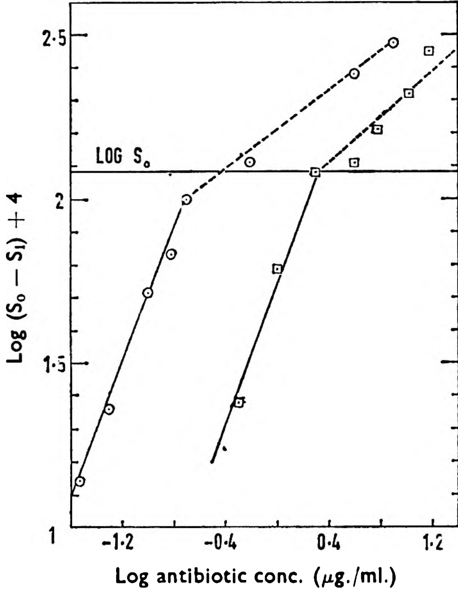

FIG. 2. Relationship between log ($S_o - S_i$) and log concentration of tetracycline or chloramphenicol for *E. coli*. S_o and S_i are growth rates in the presence of zero and i^{th} concentrations of antibiotic. ○ Tetracycline. □ Chloramphenicol.

When positive values of S_i were plotted against antibiotic concentration for both antibiotics it was observed that the slope of the straight line for tetracycline was greater than that for chloramphenicol by a factor of 7·5. This implies a difference in potency over this range. The phenomenon is illustrated in Fig. 3 by reducing the actual chloramphenicol dose by a factor of 7·5. Then the data for the two antibiotics can be accurately represented by one straight line.

Effect of Combinations of Antibiotics

Equipotent solutions of each antibiotic were made. These were then mixed in graded proportions using 100, 85, 65, 50, 35, 15 and zero per cent tetracycline solution and the residual percentage of chloramphenicol solution. This process was repeated to give seven graded, equipotent

mixtures for each of five different antibiotic equivalent concentrations. Thirty-six replicate cultures of logarithmic phase cells were incubated for a predetermined time at 37·5° when thirty-five of them were inoculated with one of the antibiotic mixtures. The slopes for the initial straight line part of the curves for the plot of logarithm colony count against time for each of the antibiotic mixtures are illustrated in Fig. 4 where the value for $(S_0 - S_i)$ is plotted against the percentage of antibiotic in the combination.

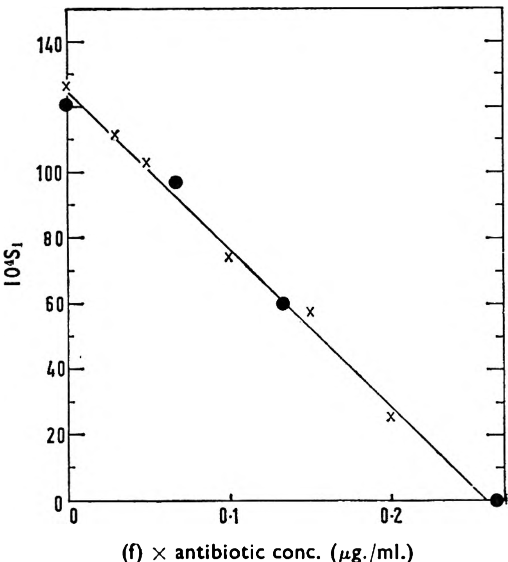

FIG. 3. Effect of tetracycline or chloramphenicol concentration upon the pseudo first order rate constant (S_i) for growth of *E. coli.* ×—Tetracycline (f) = 1. ●—Chloramphenicol (f) = 1/7·5.

DISCUSSION

The rates of growth of *E. coli* in the presence of chloramphenicol (Fig. 1) and tetracycline (Brown and Garrett, 1963) are decreased as functions of the concentrations of the antibiotics. When there is a finite increase in viable organisms the plot of the logarithm of the number of viables by colony counting, log X, against time, t, is linear (Fig. 1) so that the expression

$$X = X_0 e^{kt} \quad \ldots \quad (1)$$

is fully descriptive of the exponential growth of X_0 microorganisms from the initial time of inoculation or of antibiotic addition.

The corresponding logarithmic expression applicable to the plots of Fig. 1 is

$$\log X = S_i t + \log X_0 \quad \ldots \quad (2)$$

in the presence of an ith concentration of antibiotic where $S_i = k/2{\cdot}303$, k min.$^{-1}$.

The pseudo rate constant, S_l, is a linear function of the antibiotic concentration, D in μg./ml. for all positive values of S_l (Fig. 1 and 3),

$$S_l = S_o - k_A D_A \quad .. \quad .. \quad .. \quad (3)$$

where the subscripts represent the particular antibiotic A and where $S_o = 1{\cdot}20 \times 10^{-2}$ is 2·303 times the rate constant $k = k_o$ (min.$^{-1}$) for the growth of *E. coli* in the absence of antibiotic, $D_A = O$. The specific rate constants for antibiotic concentration effects are $k_T = 4{\cdot}81 \times 10^{-2}$ for tetracycline and $k_c = 6{\cdot}42 \times 10^{-3}$ for chloramphenicol.

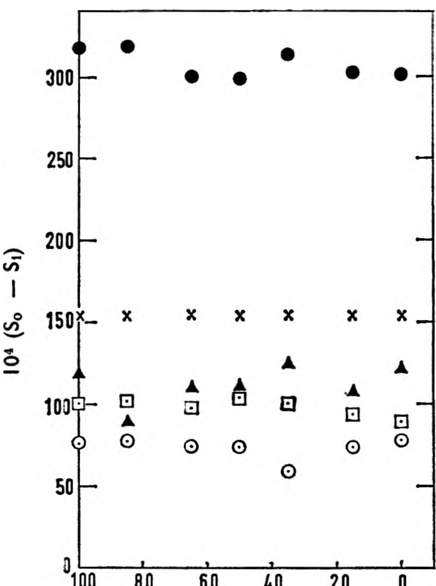

FIG. 4. Relationship between $(S_o - S_l)$ and proportion of antibiotic in a mixture of tetracycline and chloramphenicol for equivalent concentrations indicated.
(S_o and S_l) are growth rates in presence of zero and i^{th} concentration of antibiotic 7·5 μg. chloramphenicol has potency equivalent to 1 μg. tetracycline when measured separately against *E. coli*.) ●, 4 μg./ml. ×, 0·6 μg./ml. ▲, 0·2 μg./ml. □, 0·15 μg./ml. ○, 0·1 μg./ml.

If additivity of action of combined antibiotic concentrations on rates of *E. coli* growth are postulated it can be predicted for our system that

$$\begin{aligned} S_l &= S_o - k_T D_T - k_C D_C \\ &= 1{\cdot}20 \times 10^{-2} - 4{\cdot}81 \times 10^{-2} D_T - 6{\cdot}42 \times 10^{-3} D_C \quad .. \quad (4) \end{aligned}$$

where D_C and D_T are the concentrations of chloramphenicol and tetracycline respectively in μg./ml.

The substitution of equation (4) into equations (1) or (2) permits the prediction of numbers of viables at any time in the presence of various

ratios of the combined antibiotics for an initial viable count of 10^6 organisms/ml. at 37·5°.

The invariance of the exponential change of *E. coli* viables with varying ratios of chloramphenicol to tetracycline, so calculated that the potency should be equivalent on the basis of equation (4) (see Fig. 4), is confirmation of the additivity of the antimicrobial effects for these two antibiotics. Some variation is evident but not sufficient to indicate a uniform decrease of growth rate as the proportion of any one antibiotic reduces. A reduction in the rate of growth of the organism in the presence of equipotent antibiotic mixtures when compared to the effect of either antibiotic alone would have indicated synergism. Conversely, an increase in rate with the mixtures as compared to that with either antibiotic alone would have indicated antagonism (Garrett, 1958).

At $S_i = 0$ or less, a non-linearity in the plot of logarithm viables against time subsequently develops for the antibiotics separately (Fig. 1, Brown and Garrett, 1963) and in combination. This could be attributed to consumption of the antibiotic, the development of resistant mutants and/or the presence of antibiotic-resistant strains in the original inocula.

Notwithstanding this phenomena, the curves are initially linear and a slope can be obtained from a tangent to a curve at the time of antibiotic addition.

The chosen equipotency, 7·5 weight units of chloramphenicol equipotent to 1 weight unit of tetracycline hydrochloride was quite satisfactory for all values of S_i. This is evident from the fact that no significant variation in initial rate of microorganism increase or decrease occurs for any equipotent combination of antibiotics calculated on this premise (Fig. 4). On a molecular weight basis this means that tetracycline is about 11 times more active than chloramphenicol, molecule for molecule. This is not inconsistent with the molar potency ratio of 5·5 obtained by Ciak and Hahn (1958) when it is considered that they did not use tetracycline *per se*, they used a different strain of organism with a different medium and measurements were made turbidimetrically.

The rate of change of the number of viable organisms with time may be a function of the growth rate and the antibiotic concentration (Garrett, 1958), viz.,

$$(1/2{\cdot}303)\, dX/dt = S_i X = (S_0 - k_C D_C^m - k_T D_T^n)X \quad .. \quad (5)$$

an equation which integrates to equations 1 and 2, a rational explanation therefore for the apparent first order growth rates of *E. coli* in the presence of these antibiotics is that normal generation rate, S_0, competes with the removal of viable microorganisms by a rate determining attack of antibiotic molecules on the microorganism. The dependence of the slope S_i on the first power of the antibiotic concentrations (Fig. 3) for $S_i > 0$ permits the assignment m=n=1 in Equation 5 and implies the inactivation of one microorganism by one molecule of antibiotic.

An alternative method of evaluation is to subtract the slope of the log viable — time plot, S_i, for any given i^{th} dose of antibiotic, from the slope S_0 of the same system with no antibiotic. It follows from equation

(3) if the rate dependency on a power, m, of the antibiotic concentration is permitted, that

$$\log (S_o - S_i) = m\log D_A + \log k_A \quad .. \quad .. \quad (6)$$

and a plot of $\log (S_o - S_i)$ against $\log D_A$ should be linear and of a slope of positive m. From such plots (Fig. 2) for both chloramphenicol and tetracycline it is apparent that the decrease in growth rate with antibiotic concentration is a function of the first power of the concentration of these antibiotics when there is a net increase in numbers of micro-organisms, $S_i > 0$. When "kill" occurs with higher antibiotic concentrations, in the sense that there is a decrease in colony counts with time, the initial rates of such antimicrobial action are dependent on a fractional power of the antibiotic concentration.

The plots of Fig. 2 for $\log (S_o - S_i) > \log S_o$; $S_i < 0$ permit a reasonable estimate of the slope, m. Thus, the rate dependence for kill is to the 1/3 power of the concentrations of each antibiotic, although ignoring one value of the chloramphenicol data would permit m to approach 1/2 for this antibiotic.

The important conclusion, to be drawn, however, is that although diminution of positive growth rate is a function of the first power of the antibiotic concentration, the antibiotic is significantly more effective in terms of this postulated reduction in numbers of organisms capable of reproduction, i.e., "kill" in the system. This implies a difference in mechanism of action in that inhibition of growth rate may not necessarily "kill" in the same sense as with a net loss of viables. An investigation and comparison of total microorganism counts (viable and non-viable) with colony counts (viables) is needed to clarify these phenomena, to validate the basic postulates of equation 5 and to differentiate between the two possible modes of action in the change in microbial growth rates over the entire concentration ranges of these antibiotics.

The additive action of chloramphenicol and tetracylcine on *E. coli* as proposed by Ciak and Hahn (1958) on turbidimetric measurement is confirmed by our kinetic treatment of viable counting and is certainly consistent with their hypothesis of concurrent blocking of different anabolic pathways which jointly contribute to protein synthesis. However, on these kinetic grounds alone it is not possible to disregard the hypothesis that the mechanisms of action of these antibiotics are identical and based on the reaction of the drugs with the same biological site (Garrett, 1958).

Acknowledgement. The authors thank Mr. George Perry for excellent technical assistance.

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The paper was presented by DR. BROWN.

Short Communication

THE USE OF MEMBRANE FILTERS IN THE ENUMERATION OF DAMAGED *ESCHERICHIA COLI*

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IN a previous communication (Harris and Richards, 1958) the authors showed that *Escherichia coli* cells which had been treated with phenol gave lower counts on membrane filters than on nutrient agar. They suggested that the cause of this discrepancy was either the inability of the membrane to allow diffusion of essential growth requirements, or the presence of toxic materials in the environment of the cells. Gaspar and Leise (1956) reported that *Pasteurella tularensis* was inhibited by the ink used for marking the grids on membrane filters and in the absence of grids the recovery was as good as or better than that on nutrient agar. The effect was not observed with *Pasteurella pestis*, *Salmonella typhi* or *Vibrio comma*.

Therefore, the possible effects of the grid imprint material on the recovery of phenol-treated *E. coli* have been investigated. Further, the possibility that the membranes contain other toxic materials has been studied and membranes from three different manufacturers have been compared for their ability to cultivate phenol-treated *E. coli*.

The materials and methods employed were those described by Harris and Richards (1958) with the following additions.

Organism and its treatment with phenol. Only one strain of *E. coli* (type I, 44°+, IMViC++−−) was used, namely strain II of Harris, Richards and Whitefield (1961).

The growth from a 24 hr. agar culture, suspended in distilled water, was exposed to 1 per cent w/v of phenol to give a mortality greater than 90 per cent, and survivors were counted after diluting 10^4 times in distilled water.

Membrane filters and counting technique. Filters (8 cm. diameter) from three different manufacturers were used; Courtaulds Ltd. (Oxcid) with and without the grid imprint but from the same batch; Millipore Filter Corporation, Hydrosol Assay (HA) grade white Millipore filter with grids; Membranfiltergesellschaft (MFG), Coli 6 grade without grid markings. When washed membranes were required these were boiled gently with 100 ml. of distilled water, dried and sterilised as usual. In some experiments the filter washings were incorporated in nutrient agar so that 20 ml. of nutrient agar contained the washings from a single filter.

The filters were sterilised by autoclaving, dried overnight at 40°, and supported for counting either on Whatman No. 17 filter pads which had been treated similarly or, less often, on nutrient agar.

The nutrient broth used contained 16 g./litre of "Oxoid" Lab-Lemco granules (CM 15) and was set with 15 g./litre of New Zealand agar when

required. All counts were done by the surface viable method using 10 replicate drops and incubating for 24 hr. at 37°.

Results and Discussion

Effect of grid markings. Both untreated and phenol-treated cells were counted on nutrient agar and on membrane filters with and without the grid markings. Using untreated cells no differences in the counts were observed under the different conditions. The results with phenol-treated cells are presented in Table IA, which shows that very poor recoveries were

TABLE I

Viable counts of phenol-treated *E. coli* on nutrient agar and on oxoid membrane filters

A. The effect of printed grids			B. The effect of washing the filters		
Mean viable count/drop			Mean viable count/drop		
Nutrient agar (control)	Membrane filters		Nutrient agar (control)	Membrane filters	
	With grid	Without grid		Unwashed	Washed
52·8	18·6	—	20·7	—	7·4
67·8	24·3	—	67·6	—	31·6
86·1	27·7	—	61·3	17·7	30·5
54·4	22·7	—	85·1	38·2	33·2
88·1	56·5	—	4·1	0·1	0
121·0	63·4	50·2	82·2	21·2	31·6
114·5	80·3	65·9	92·3	51·1	47·6
65·9	33·0	31·7	125·0	55·9	64·7
125·7	49·4	61·7	20·8	3·9	3·1
86·8	59·4	34·9	27·5	6·1	8·8
19·9	3·0	2·0			
127·3	96·0	47·7			
100*	48*	41*	100*	30*	36*

*Mean response (per cent of control).

obtained on the membrane filters, thus confirming the results of Harris and Richards (1958). Both types of membrane filter gave significantly lower counts than did the nutrient agar (P, <0·001 in both cases). The membrane filter without the grid markings usually gave slightly lower counts than the membrane with the grids but the differences were not significant ('*t*', 2·13; d.f., 6; P, 0·05–0·1). Thus the printed grids were not implicated in the low counts obtained on the membrane filters.

Tests for the presence of toxic materials in the membrane filters. The possibility that water soluble toxic materials are present in the membrane filters was investigated using washed membranes. Untreated cells cultured on nutrient agar and on the membrane filters gave no differences in count. The results using phenol-treated cells are presented in Table IB. Both washed and unwashed membrane filters gave significantly lower counts than did the nutrient agar (P, <0·001). The washed membrane filters gave a slightly higher mean count than did the unwashed ones, but the difference was not significant ('*t*', 1·02; d.f., 7; P, 0·3–0·4). The inclusion of washings in nutrient agar did not influence the viable counts of phenol treated cells. Thus any inhibitory factor, if present, either was not water soluble or did not diffuse freely out of the membrane filter and so was

concentrated in it, in the immediate environment of the damaged cells. One difficulty in the interpretation of the results with washed membrane filters was caused by the failure of the drops of inoculum to spread as they did on unwashed filters, and the influence of this on the counts cannot be determined.

TABLE II

VIABLE COUNTS OF *E. coli* ON THREE DIFFERENT TYPES OF MEMBRANE FILTERS AND ON NUTRIENT AGAR

Treatment	Mean viable count/drop						
	Nutrient agar (control)	Membrane filters					
		Oxoid		Millipore		MFG	
		N.A.**	F.P.**	N.A.	F.P.	N.A.	F.P.
None	35·8	32·1	33·3	—	—	—	—
	40·8	36·8	39·8	35·6	25·0	33·4	21·5
	39·1	36·5	32·3	34·7	25·9	36·4	35·7
	34·2	36·1	33·1	33·6	31·3	35·0	31·0
	100*	95*	93*	91*	73*	92*	78*
Phenol	205·0	—	—	83·0	70·3	—	84·0
	215·2	145·4	—	90·2	26·9	88·4	—
	204·6	134·4	—	83·8	34·3	93·8	48·7
	128·4	73·0	33·0	—	3·1	—	1·8
	210·8	110·6	—	80·6	53·6	81·8	43·7
	136·6	94·4	—	62·1	24·7	53·2	12·9
	141·6	83·6	—	56·1	12·5	42·1	14·1
	16·9	1·3	0·4	0·3	0·4	0·4	0·3
	100*	54*	14*	33*	15*	33*	15*

*Mean response (per cent of control).

**Membrane filters incubated on: N.A., nutrient agar; F.P., No. 17 filter paper pad impregnated with broth.

Comparison of three different types of membrane filter. Untreated and phenol-treated cells were cultivated on nutrient agar and on the three different membrane filters, the filters being used both on the surface of nutrient agar and on filter paper pads, and the results are given in Table II. Using untreated cells none of the counts was significantly different from the nutrient agar control. In contrast with these results, Hess and Speiser (1959) using MFG Co 5 membrane filters observed that counts from dilute suspensions of *Pesudomonas pyocyanea* were significantly higher than with pour plates, though the differences were small.

With phenol-treated cells cultured on the membrane filters there were substantial reductions in the counts (Table II), and both the Millipore and the MFG filters gave even fewer colonies than the Oxoid, whether they were incubated on the surface of the nutrient agar or on the filter paper pads. The differences between the counts on nutrient agar and on the membrane filters were significant in all cases ($P, <0{\cdot}01$).

Thus it is clear that the poor performance of membrane filters relative to nutrient agar reported by Harris and Richards (1958) was not an isolated occurrence with one type of filter only and may be associated generally with the membrane filter technique. In this connection Lightbown (1962) found poor recoveries of *Bacillus subtilis* on membrane filters under some conditions.

Postgate and Hunter (1962) have observed that membrane filters contained substances which accelerated the death of starved bacterial populations, and it was suspected that the membrane filters contained surface-active substances, since drops spread much less on washed Oxoid filters than on unwashed ones. The presence of anionic surfactants in the Millipore and Oxoid membrane filters (but not in the MFG) was confirmed by surface tension measurements on the washings (about 55 to 60 dynes/cm.) and by dye transference tests.

Further, preliminary experiments using suspensions treated with cetrimide (0·005 per cent w/v) showed that counts on unwashed membrane filters were similar to those on nutrient agar, while washed membrane filters gave lower counts. This phenomenon may have resulted from neutralisation of the cetrimide carried over in the inoculum by surfactants present in the filter. The results, however, are insufficiently conclusive to warrant a definite opinion about the reasons for the low counts of cells treated with phenol on the membrane filters. Since membrane filters can give poor recoveries of damaged cells, the use of such filters in sterility testing and similar applications should be approached cautiously.

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The paper was presented by DR. RICHARDS.

THE INFLUENCE OF THE NATURE OF THE RECOVERY MEDIUM ON THE APPARENT VIABILITY OF PHENOL-TREATED BACTERIA

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Suspensions of *Escherichia coli* and *Staphylococcus aureus*, untreated and treated by exposure to phenol, *o*-cresol or *p*-chloro-*m*-cresol, were counted on various types of media. While the counts from untreated suspensions were not affected by the nature of the medium, those from treated suspensions were influenced markedly. There was no correlation between the nutritional status of a medium and its suitability for the cultivation of the treated organisms. Although both bacterial species gave similar patterns of response and ranked the media in roughly the same order of suitability, *E. coli* was the more responsive species. Generally, the order of suitability was: meat extracts, yeast extracts, meat extract-peptone broth, peptones (all the single ingredients 1 per cent, pH 7; media solidified with New Zealand agar). Although supplementation of the meat extract-peptone control medium, especially with yeast extracts, led to higher counts from treated suspensions, dilution of the extract broth also gave similar results. Further, a fresh infusion broth was superior both to the extract broth and to a digest medium, for counting treated bacteria. The deficiencies of the extract broth were due partly to the phosphate precipitation procedure used in its preparation. The results indicate that when media suitable for the growth of damaged bacteria are required, they should be selected without recourse to preconceived ideas about composition, concentration or method of preparation.

MANY types of media are used for the growth of bacteria, numerous complex materials being employed as enrichments to promote the growth of exacting species (Knight, 1936; Oxoid Manual, 1961; Difco Manual, 1953) and Jacobs and Harris (1960) have drawn a parallel between such organisms and damaged bacteria. The importance of the nature of the medium used for the growth of damaged bacteria and bacterial spores has been emphasised by Nelson (1943) and the value of enrichments has also been stressed (Curran and Evans, 1937; Thompson, Mefferd and Wyss, 1951). In this connection, yeast extracts have been found to reverse inhibition by acriflavine (McIlwain, 1941) and penicillin (Chattaway, Hall, Happold and Holdsworth, 1949). Also, several authors observed that recoveries with frozen cells were higher in rich than in minimal media (Straka and Stokes, 1959; Nakamara and Dawson, 1962; Postgate and Hunter, 1963). Although Needham (1947) used a simple peptone water as a growth medium after disinfectant testing, the suitability of this was not determined using damaged cells.

However, the selection of media and their composition is primarily empirical, and it was thought useful to examine in detail the performance of a wide range of materials for the cultivation of damaged bacteria.

Materials and Methods

Throughout this paper, all percentages are given as weight in volume (w/v) unless otherwise stated.

Organisms and bactericidal treatments. Two species were used: a laboratory strain of *Escherichia coli* type I (44° +), described previously (Jacobs and Harris, 1960), and *Staphylococcus aureus* NCTC 4736. They were grown on nutrient agar slopes for 24 hr. at 37° and suspensions of washed cells containing about 10^9 viable cells/ml. were prepared.

To 4 ml. of bactericide at 20° ± 0·5 was added 1 ml. of suspension, and conditions were such that the final concentrations in the reaction mixtures were: phenol 1 per cent, *o*-cresol 0·4 per cent, *p*-chloro-*m*-cresol (PCMC) 0·075 per cent. The reaction was stopped when desired by diluting at least 200 times, and survivors were counted. A wide range of contact times and dilutions were used to allow for day to day variations in the responses of the suspensions, but conditions were such that mortalities were of the order of 90 to 99 per cent, and were often greater.

Media. The extract broth employed routinely and as control medium contained peptone (Oxoid no. L 37), 10 g.; Lab-Lemco, 10g.; sodium chloride, 5 g.; tap water to 1 litre: the normal method of phosphate precipitation was used.

Single medium constituents were used as simple 1 per cent solutions in tap water and were processed only by adjustment to pH 7·0, sterilisation and the addition of agar. For some experiments Hartley's broth and an infusion broth were prepared using established procedures (Mackie and McCartney, 1953), although the infusion broth was prepared both with and without the phosphate precipitation procedure.

Unless stated otherwise, media were solidified using 15 g./litre of New Zealand agar, though 25 g./litre of powdered Japanese agar was used for some experiments. All media were sterilised by autoclaving for 20 min. at 15 lb./in.^{2}

Colony counts and presentation of results. Counts were done by the surface viable method (Miles and Misra, 1938) using 5 replicate 0·02 ml. drops, and colonies were counted after 48 hr. or 24 hr. at 37°, using a lens, the 48 hr. count being used wherever possible since experience showed that numbers were then maximal. Generally at least 2 trials were done with untreated organisms, and at least 3 trials with suspensions exposed to all 3 bactericides, using each batch of media; 2 or 3 batches of media were often used. Thus the full results are too extensive for complete presentation and, for clarity, only mean values are given in the Tables. In each trial the counts were expressed as a percentage of the control count, and the mean relative responses computed as the means of these percentages. Since this work was completed the wide variation in response between individual trials, and between batches, has been explained (Harris, Richards and Whitefield, 1961): so too much emphasis should not be placed on the magnitudes of the observed responses, which indicate only the effects which it is possible

to obtain in such experiments. However the data rank the media reliably in order of suitability. The difficulties of applying conventional statistical procedures to such data have been discussed by Harris and Jacobs (1960).

RESULTS

The Growth of Bacteria Damaged by Phenols on a Range of Medium Constituents

Preliminary trials. The results of initial experiments with a limited range of materials indicated clearly that, although untreated suspensions of both species gave similar counts on all the media, counts from phenol-treated suspensions varied widely and were highest on simple meat extract media, less on yeast extracts and lowest on peptone waters, the effects being more marked with *E. coli* than with *Staph. aureus.* Accordingly, the experiments were repeated using both species damaged by all three bactericides, and the results (Table I) confirmed the earlier findings. Although the two species behaved somewhat differently, in both cases Bacto-Beef Extract gave the highest counts and Bacto-Peptone the lowest, the control extract broth being only slightly better than the poorest medium.

TABLE I

THE COUNTS OF BACTERIA TREATED WITH PHENOLS ON A RANGE OF MEDIUM CONSTITUENTS

Medium†	Mean relative response* after treatment					
	E. coli			*Staph. aureus*		
	Phenol	*o*-Cresol	PCMC‡	Phenol	o-Cresol	PCMC‡
Oxoid Peptone	450	225	909	150	100	117
Bacto-Peptone	50	75	298	0	100	50
Lab-Lemco	2,800	3,450	14,500	375	225	300
Bacto-Beef Extract ..	26,450	36,150	32,700	850	1,030	917
Oxoid Yeast Extract ..	350	375	6,690	700	500	317
Bacto-Yeast Extract ..	375	1,150	14,390	1,250	600	500

* Relative to extract broth (Lab-Lemco, Oxoid Peptone) as 100.
† All 1 per cent in tap water, pH 7·0; media set with New Zealand agar.
‡ PCMC, *p*-chloro-*m*-cresol.

Results with a wider range of medium constituents. These are given in Table II. Once again, counts of untreated cells of both species were similar on all media, while counts from treated suspensions varied considerably. Bacto-Peptone was the least suitable medium, whatever the organism or treatment, and all the peptones, casein hydrolysate and malt extract gave low counts relative to the control. Materials giving the highest responses in this group were casein hydrolysate with *E. coli* and Bacto-Tryptone with *Staph. aureus*, and with these materials counts approached those of the control. In contrast to these results, counts on all the meat and yeast extracts were substantially higher than on the extract broth, except with *Staph. aureus* on Oxoid Liver Extract where results were rather variable. Results with *E. coli* were especially striking with this batch of media.

TABLE II

THE COUNT OF UNTREATED AND PHENOL-TREATED BACTERIA ON A WIDE RANGE OF MEDIUM CONSTITUENTS

	Mean relative response* after treatment							
	E. coli				*Staph. aureus*			
Medium†	Un-treated	Phenol	*o*-Cresol	PCMC	Un-treated	Phenol	*o*-Cresol	PCMC
Peptones, etc.								
Oxoid Peptone	87	5	10	5	101	46	28	39
Bacto-Peptone	91	< 1	1	< 1	100	16	19	17
Bacto-Tryptone	98	31	22	15	108	93	80	49
Eupepton No. 2	88	2	7	4	107	28	41	43
Casein Hydrolysate	93	47	105	74	107	52	74	45
Malt Extract	81	49	11	19	102	56	29	24
Meat and Yeast Extracts								
Lab-Lemco	106	49×10^4	45×10^4	16×10^4	106	650	746	411
Bacto-Beef Extract	106	50×10^4	40×10^4	13×10^4	118	660	623	440
Wilson's Beef Extract	93	41×10^4	36×10^4	12×10^4	114	736	763	367
Jardox	102	54×10^4	44×10^4	21×10^4	103	690	719	448
Oxoid Liver Extract	98	1750	688	2443	110	144	177	123
Bacto-Yeast Extract	93	38×10^4	35×10^4	16×10^4	100	245	359	203
Oxoid Yeast Extract	103	15×10^4	20×10^4	8×10^4	113	249	256	184
Yeastrel	92	28×10^4	34×10^4	14×10^4	108	618	599	353
Marmite	89	33×10^4	31×10^4	16×10^4	112	148	421	228

* Relative to extract broth as 100.
† All 1 per cent in tap water, pH 7; all media set with New Zealand agar. Sources as follows: Lab-Lemco and Oxoid products, Oxo Ltd., London, E.C.4; Bacto Products, Baird and Tatlock Ltd., Chadwell Heath, Essex; Eupepton No. 2, Casein Hydrolysate (vitamin free) and malt extract, Allen and Hanbury's Ltd., London, E.2; Wilson's Extract of Beef, Wilson's Meats Ltd., London, E.C.1; Jardox, Jardox Concentrated Products Ltd., London, S.E.20; Yeastrel, Brewer's Foods Supply Co. Ltd., Edinburgh, 3; Marmite, The Marmite Food Extract Co. Ltd., London, E.C.3.

Repetition of the above experiments using a second batch of media gave a similar pattern of results, though the magnitude of favourable responses was substantially less, especially with *E. coli* with which the meat extracts gave mean counts 3 to 4 times those on the control, while those with *Staph. aureus* were about twice the control count. The yeast extracts gave intermediate responses with both species.

Results with Japanese agar. It was known that media solidified with Japanese agar gave substantially higher counts than those prepared with New Zealand agar (Jacobs and Harris, 1961), and experiments were repeated using media solidified with the former material (Table III). This time, counts with treated *Staph. aureus* were lower than on the

TABLE III

THE COUNTS OF UNTREATED AND PHENOL-TREATED BACTERIA ON A RANGE OF MEDIUM CONSTITUENTS SOLIDIFIED WITH JAPANESE AGAR

	Mean relative response* after treatment							
	E. coli				*Staph. aureus*			
Medium†	Un-treated	Phenol	*o*-Cresol	PCMC	Un-treated	Phenol	*o*-Cresol	PCMC
Oxoid Peptone	100	18	10	5	100	13	46	25
Bacto-Peptone	93	13	64	62	93	16	34	27
Lab-Lemco	95	1,865	530	475	109	78	92	23
Bacto-Beef Extract	96	3,093	878	688	93	86	82	91
Oxoid Yeast Extract	100	935	376	311	93	71	59	78
Bacto-Yeast Extract	99	80	70	38	91	48	58	61

* Relative to extract broth as 100.
† All 1 per cent in tap water, pH 7.

control on all the single ingredient media, but with treated *E. coli*, Bacto-Beef Extract, Lab-Lemco and Oxoid Yeast Extract again gave mean counts higher than did the control extract broth, while Bacto-Yeast Extract, Bacto-Peptone and Oxoid peptone gave lower counts. Despite the smaller responses relative to the control, however, the materials were still graded in approximately the same order of suitability for revival.

The Influence of the Method of Medium Preparation on the Counts of Damaged Bacteria

The results reported above showed that a medium prepared from Oxoid peptone and Lab-Lemco almost always gave lower counts of damaged cells than one containing Lab-Lemco only, and this was sometimes true also with the simple peptone water medium. It seemed possible that the phosphate precipitation procedure used in the preparation of the extract broth could have been responsible for this phenomenon. Accordingly, counts of untreated and treated suspensions of both species were done on 3 media solidified with agar: viz., the standard extract broth; a simple solution of 1 per cent Lab-Lemco plus 1 per cent Oxoid peptone, with no pH adjustment; the latter adjusted to pH 7 before autoclaving, no phosphate precipitation being done.

TABLE IV

THE EFFECT OF PHOSPHATE PRECIPITATION TREATMENT OF A MEDIUM ON THE COUNTS OF UNTREATED AND PHENOL-TREATED BACTERIA

Treatment of medium† and batch number		Mean relative response* after treatment							
		E. coli				*Staph. aureus*			
		Un-treated	Phenol	*o*-Cresol	PCMC	Un-treated	Phenol	*o*-Cresol	PCMC
Phosphate precipitation ..	—	100	100	100	100	100	100	100	100
None‡	1	96	3,960	1,740	2,820	92	214	159	168
	2	100	71	81	110	90	38	55	63
	3	106	41	84	57	100	4	41	52
Adjust to pH 7·0	1	100	971	810	1,210	102	153	134	132
	2	103	183	148	192	104	127	142	137
	3	124	666	220	235	104	135	122	149

* Relative to standard extract broth, prepared with phosphate precipitation, as 100.
† All media 1 per cent each Lab-Lemco and Oxoid Peptone, 0·5 per cent NaCl; solidified with New Zealand agar.
‡ Batch 1, pH 6·2; batch 2, pH 5·9; batch 3, pH 5·5.

The mean results of trials with 3 batches of each medium are given in Table IV and show that the medium adjusted to pH 7, but on which no phosphate precipitation had been carried out, always gave higher counts of damaged cells than did the control. Once again, responses with *E. coli* were considerably larger than those with *Staph. aureus*. On the medium prepared without either phosphate precipitation or pH adjustment, higher counts than on the control were obtained with batch 1, but subsequent batches gave lower counts than the extract broth. This marked difference in response between batches could have been due to the variation in pH, which declined from 6·2 in batch 1 to 5·5 in batch 3. The reason for this pH variation is not known.

Digest and infusion media. Freshly prepared media, and especially those prepared by digestion, are generally accepted as superior to those containing prepared ingredients. The results of counting untreated and treated suspensions on extract, digest and infusion media are given in Table V. Since it was known that phosphate precipitation damaged the extract medium, the infusion broth was prepared both using and omitting this procedure, both types being adjusted to pH 7·2, to check whether damage resulted with this medium also. When phosphate precipitation was not used, a precipitate was produced during sterilisation, but this did not interfere with counting by the surface viable technique.

TABLE V

THE COUNTS OF UNTREATED AND PHENOL-TREATED BACTERIA ON EXTRACT, DIGEST AND INFUSION MEDIA

Type of broth and batch number		Mean relative response* after treatment							
		E. coli				*Staph. aureus*			
		Un-treated	Phenol	*o*-Cresol	PCMC	Un-treated	Phenol	*o*-Cresol	PCMC
Infusion, no phosphate precipitation	—	100	100	100	100	100	100	100	100
Infusion, with phosphate precipitation	1	—	—	—	—	—	—	—	—
	2	94	61	58	50	113	109	72	72
Hartley's Digest	1	104	<1	<1	<1	103	35	50	62
	2	96	7	13	2	97	89	50	29
Extract (standard)	1	99	<1	<1	<1	104	55	62	81
	2	97	22	29	21	103	91	95	69

* Relative to infusion medium, prepared without phosphate precipitation, as 100. All media set with New Zealand agar.

All four media gave similar counts with untreated suspensions but, with treated ones the infusion medium prepared without phosphate precipitation always gave substantially higher counts than the other media. Results are therefore expressed using this medium as a control in place of the standard extract broth. Previous results were confirmed, in that responses were more marked with treated *E. coli* than with *Staph. aureus* and phosphate precipitation usually impaired the infusion medium. Although the digest medium gave a substantially greater bulk of growth of both species than did the extract medium, counts after exposure to phenols were lower on the former than on the latter.

Supplementation. Routine media are often supplemented with yeast extracts and other materials to improve their growth capacity, especially for exacting species. Various supplements were added singly to the standard extract broth and the counts of phenol-treated suspensions on the resultant media, none of which affected the counts of untreated cells, are shown in Table VI. Treated suspensions however yielded counts higher on supplemented media than on the control. Yeastrel and Bacto-Yeast Extract were the most favourable additives, while responses to the addition of casein hydrolysate were small.

TABLE VI

THE INFUENCE OF SUPPLEMENTATION OF THE STANDARD EXTRACT BROTH ON THE COUNTS OF BACTERIA DAMAGED BY PHENOLS

Supplement†	Mean relative response* after treatment					
	E. coli			*Staph. aureus*		
	Phenol	*o*-Cresol	PCMC	Phenol	o-Cresol	PCMC
Bacto-Yeast Extract	482	522	333	170	160	160
Oxoid Yeast Extract	103	145	162	142	124	117
Marmite	182	370	256	162	149	128
Yeastrel	479	2,263	548	164	155	127
Casein Hydrolysate	109	110	134	129	114	125
Malt Extract	581	812	406	196	170	132
Oxoid Liver Extract	280	170	244	142	137	134

* Relative to unsupplemented extract broth as 100, mean of 3 batches of media, all set with New Zealand agar.
† 1 per cent of the substance was added to the extract broth after phosphate precipitation and adjusted to pH 7·0.

No favourable effects were observed when media were supplemented either with colloidal materials, e.g., 1 per cent horse serum, egg albumen, acacia or starch, or, despite the beneficial effect of Malt Extract, with a range of fermentable carbohydrates.

Medium concentration. Since all the supplemented media were more concentrated than the control, and the medium used in the standard Rideal-Walker test (B.S. 541, 1953) is twice the concentration used routinely here, the effects of medium concentration were investigated. Portions of a double strength extract broth were diluted with distilled water to give broths of normal strength and one third normal strength. The results with two batches of these media are given in Table VII.

TABLE VII

THE INFUENCE OF MEDIUM CONCENTRATION ON THE COUNTS OF UNTREATED AND PHENOL TREATED BACTERIA

Concentration of broth and batch number	Mean relative response* after treatment							
	E. coli				*Staph. aureus*			
	Un-treated	Phenol	*o*-Cresol	PCMC	Un-treated	Phenol	*o*-Cresol	PCMC
Double strength .. 1	100	<1	1	<1	102	71	60	49
2	108	12	1	<1	105	50	67	66
One-third strength 1	103	688	3,935	671	105	328	243	174
2	100	4,000	3,600	4,875	106	152	156	136

* Relative to normal strength extract broth as 100. All media set with New Zealand agar.

Contrary to expectation, counts of damaged organisms increased with increasing dilution; strikingly higher counts being obtained on the most dilute medium with treated *E. coli*, and although responses with treated *Staph. aureus* were smaller, they were still substantial.

DISCUSSION

Two general trends are clear from the above results; that untreated suspensions of *E. coli* and *Staph. aureus* always gave similar counts

whatever the type of medium employed, whereas treated cells were markedly sensitive to their environment, and that treated *E. coli* cells were considerably more responsive to revival than those of *Staph. aureus*. Both of these phenomena have been observed previously (Jacobs and Harris, 1960; 1961).

There are numerous reports on the restraining action which organic matter has on bactericidal action (e.g., Chick and Martin, 1905; Klarmann, Shternov and von Wowern, 1929; Garrod, 1935), and Winslow and Brooke (1927) attributed the abolition by dilute broth of the death of bacteria in distilled water to a protective action of colloidal matter. In this connection, the protection of bacteria by young, heat killed organisms (Lange, 1922) is also relevant. However, no evidence was obtained here that colloidal material favoured revival. However, if death from phenol is due partly to leakage of metabolites from cells (Gale and Taylor, 1947; Salton and Alexander, 1950), then replacement of leaked materials by nutrients from the medium may also be important. It is difficult to decide what substances may be most valuable for revival, but it is known that amino-acids, nucleic acid residues and growth factors may be liberated from damaged cells (Cook and Cronin, 1941; Gale and Taylor, 1947; Loofbourow, 1947; Loofbourow and others, 1947; Salton and Alexander, 1950). Therefore, it is not surprising that meat and yeast extracts, which are rich in such materials, were more favourable to revival than were peptones, which are relatively poor in them. The importance of yeast nucleotides in promoting the viability of cells other than bacteria has been demonstrated for tissue cultures (Lesfargues and Delaurey, 1947). However, media which contained abundant nutrients, and which gave excellent growth of untreated suspensions, often gave lower counts of damaged cells than did media of low nutrient content, so nutrient capacity alone cannot account for the observed differences. Also no support was found for the recommendations that fermentable carbohydrates should be included in the revival medium (Supfle and Dengler, 1916; Flesch, 1921).

One possibility is the occurrence in media of substances toxic to damaged cells, and this has been discussed by Jacobs and Harris (1960, 1961) who concluded that both extract broth and agar contain such substances. They showed that Japanese agar was less toxic than New Zealand, and this could explain the discrepancies between the results reported in Table III from those in Tables I and II. If the control medium prepared with Japanese agar was less toxic than the normal control with New Zealand agar, then favourable responses normally observed with other media could be masked.

Circumstances may be envisaged however, in which less rich media could give improved growth of damaged cells. The revival of such cells must depend on the relative rates of recovery and lethal processes, and there is some evidence that retardation of metabolism may result in recovery. When irradiated with X-rays, *E. coli* has been reported to recover better on less rich media (Alper and Gillies, 1958) and at temperatures below the normal optimum for the species (Hollaender,

Stapleton and Billen, 1953; Harris and Whitefield, 1964). If similar mechanisms operate with phenol treated organisms, the higher counts obtained on some media may reflect their lower nutritional status or greater toxicity.

The increased counts obtained with treated cells on the first batch of medium prepared without phosphate precipitation or pH adjustment (Table IV) indicates that acidity down to pH 6·2 was not actively harmful. However, the results must be viewed cautiously, since the improvement over the control could have resulted partly from hydrolysis of some constituents.

The main conclusion which may be drawn is that established ideas as to the suitability of a medium, and of what factors contribute to this, need reconsideration in relation to damaged cells, since existing information on media relates primarily to undamaged cells. Although there is a gradation from robust to exacting species of bacteria, it is clear that even those normally considered to be non-exacting become fastidious after damage, and situations in which damaged organisms occur are very common.

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The paper was presented by The Author.

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Short Communication

PHASE SOLUBILITY ANALYSIS: AN EVALUATION OF THE TECHNIQUE

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THE increasing prominence given to the need for reference standards has prompted an investigation of possible assay methods for assessing the purity of such substances. The advantages claimed for phase solubility analysis made this an obvious choice for examination. Our investigations arose in part from work undertaken by one of us (D.C.G.) as a member of the Synthetic Drugs Committee of the British Pharmacopoeia Commission.

Parsons, Genarro and Osol (1961) have used phase solubility analysis in assaying adrenocortical steroid reference standards. This method has been fully described by Mader (1954) but as far as we are aware has received little attention in this country. It is based on the principles of Gibbs' phase rule and involves equilibrating varying weights of solid with a fixed weight of solvent in sealed ampoules at constant temperature and pressure. When equilibrium has been established the weights of solute per gram of solvent (Solution Concentration) are determined and plotted against the weights of solid originally taken per gram of solvent (System Concentration). A phase solubility curve is obtained and the percentage total impurity is calculated from the slope of the curve immediately following the first turning point.

For a pure compound the slope will be zero whilst for a compound containing one impurity the slope (expressed as a percentage) gives the impurity. For a compound containing several impurities several turning points, each corresponding to an impurity, may be obtained. The total impurity is calculated from the slope of the curve following the first turning point whilst the individual impurities are obtained from the difference in the slopes of the curves on either side of the succeeding turning points.

Table I lists the results obtained by this and other methods of assay. It shows that, when applied to the analysis of mecamylamine hydrochloride, the method outlined in the experimental section gave results reproducible to within $\pm 0{\cdot}3$ per cent.

At this stage in our investigation it is our opinion that the sensitivity and selectivity of the technique outweigh the disadvantages of lengthy analysis time. Furthermore, we feel that this technique may be of further value not only in assessing the purity of possible reference standards, but in the detection of closely-related impurities in new synthetic drugs.

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Experimental

Various techniques have been described for this type of assay (Tarpley and Yudis, 1953; Outch, 1961; Parsons and others, 1961). The reasons underlying our choice of technique are outlined below.

(i) Neutral glass ampoules were used since the possibility of an impurity being formed during equilibration could not be discounted with soda glass. Ampoules were rinsed once with 5 per cent acetic acid, 6 times with distilled water, then dried at 105°.

(ii) Solvents or mixed solvents in which the solubility of the compound being determined is between 0·4 and 2·5 per cent, as recommended, have been found to be satisfactory (Mader, 1954). The boiling-points should ideally lie between 60–100°. It was found unnecessary to chill the ampoules during the sealing operation.

TABLE I

Compound	Solvent system and solubility at 25°	Phase solubility assay		Tetrazolium assay	Ultra-violet* assay
		per cent impurity	per cent compound	per cent compound	per cent compound
ydrocortisone B.P.	14·2 mg./g. in methanol	3·3	96·7	99·4 100·0	100·4
Hydrocortisone B.P.	14·2 mg./g. in methanol	1·7	98·3	99·4 99·0	99·3
Prednisolone†	5·96 mg./g. in benzene-methanol (95 + 5)	12·5	87·5	92·5 93·3	99·0
				Chloride assay per cent compound	
Mecamylamine hydrochloride	19·7 mg./g. in isopropanol				
Batch 1		4·6; 4·7	95·4; 95·3	100·7; 100·6; 100·7	
Batch 2 equilibratec 2 days		1·8; 1·7 1·9; 2·0; 1·8	98·2; 98·3 98·2; 98·0; 98·1	99·9; 100·1; 100·3	
Batch 2 equilibratec 10 days		2·1; 1·6	97·9; 98·4		

* Official method of the British Pharmacopoeia, 1958.
† Process sample before purification.

(iii) The period of equilibration varies from system to system. Four weeks has been used for corticosteroids, whilst 2 days has been found sufficient for mecamylamine hydrochloride.

(iv) Having separated the liquid from the solid phase, the solution concentration is determined gravimetrically after evaporation of the solvent. The method of evaporation is governed by the thermal stability of the compounds being examined. Evaporation on a steam-bath whilst a gentle stream of oxygen-free nitrogen is blown across the surface of the solvent has been found to be satisfactory for hydrocortisone in methanol, prednisolone in benzene-methanol and mecamylamine hydrochloride in isopropanol.

(v) Slopes of the curves have been calculated statistically to obtain the line of best fit. Small variations in the technique are thus averaged out and an accurate measure of the impurity obtained.

All solvents were of analytical reagent grade. Cotton Wool B.P.C. was washed with methanol and dried at 100°. Substances under investigation were all finely ground and dried *in vacuo* at room temperature over phosphorus pentoxide.

Neutral glass ampoules (20 *ml.*). Rinsed once with 5 per cent acetic acid followed by six water washings and dried at 105°.

Procedure. Transfer the required amounts of substance to eight clean, dry, tared glass ampoules, each of 20 ml. capacity, by means of a long-stemmed funnel, taking care to avoid contaminating the neck of the ampoule. Re-weigh each ampoule, add solvent (15 ml.) from a glass syringe and flame seal the ampoules; cool and re-weigh. The concentration in one of the ampoules should lie below the saturation point, whilst the system concentration in the remaining seven should be varied from just above the saturation point to five times this concentration. (Solvent systems and solubilities of the substances examined are given in Table I.)

Equilibrate the ampoules by lengthwise rotation (50 r.p.m.) in a water-bath at 25·0 (±0·1°) for the specified time. Support the ampoules vertically in the bath for a further 24 hr. to allow the solid phase to settle.

Remove a sample of solution (10 ml.) whilst the ampoules are still in the bath; use a glass syringe fitted with a 3½ in. × 17 G hypodermic needle. The needle should be fitted with a stop, to prevent the solid phase being disturbed, and the attachment end should contain a small plug of cotton wool to act as a filter. Remove the needle and immediately transfer the solution to a dried, tared (to the nearest 0·01 mg.) weighing bottle (25 ml.) fitted with a ground-glass stopper, and re-weigh. Evaporate the solution to a volume of 1 ml. as in (iv) above, remove the last traces of solvent at room temperature with oxygen-free nitrogen. Dry to constant weight in a vacuum desiccator over phosphorus pentoxide at a pressure not exceeding 5 mm. mercury.

A plot of the system concentration (X mg./g. of solvent) versus the solution concentration (Y mg./g. of solvent) should give a straight line if equilibrium has been reached.

$$\text{per cent total impurity} = \frac{100 \times \Sigma XY - \dfrac{(\Sigma X)(\Sigma Y)}{N}}{\Sigma X^2 - \dfrac{(\Sigma X)^2}{N}}$$

where N = number of ordinates of the first slope.

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Discussion

The paper was presented by Mr. Johnson. The following points were made in the discussion.

The time of equilibration might be influenced by a number of factors. It has been suggested that solid particles in contact with a liquid become surrounded by a thin film of solution and that the solid then diffuses through this film into the bulk of the liquid; the rate of this diffusion depends on the nature of the solid, the temperature, and the viscosity of the surrounding film. Particle size might also play a part since a finely divided sample sometimes has a greater solubility than one less finely divided. The extent to which supersaturation occurs would affect the time of equilibration and, in the case of the steroids, polymorphism may play a part since the same substance in the two different crystalline forms would behave as two components. The phase solubility method would fail if two components were present in the same ratio as their solubilities; this could be overcome by choosing a different solvent system or changing the temperature of equilibration. Other causes of failure were a non-ideal behaviour of the solution (avoided by working with suitably dilute solutions), the formation of solid solutions (frequently overcome by using a different solvent system) or some variation in the chemical nature of the solute due to reaction with the solvent or to decomposition. The latter possibility is indicated by an apparent over or under recovery of solute in the first ampoule. Solvents used should be of a high degree of purity and mixed solvents should be avoided if possible; the solvent system chosen should be such that the main component in the sample being examined is the first to saturate the system.

THE COLORIMETRIC DETERMINATION OF SMALL AMOUNTS OF IODIDE, WITH SPECIAL REFERENCE TO THE DETERMINATION OF IODIDE IN THE PRESENCE OF ORGANICALLY-BOUND IODINE

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A photocolorimetric method has been developed for the determination of small quantities of inorganic iodide in the presence of organically-bound iodine. The iodide-containing solution is treated with a mixed iodate-starch solution at an appropriate pH, when a stable blue colour is produced. The relationship between concentration of iodide and optical density is linear. The effect of various salts on the reaction has been investigated; the analytical application of the method to some iodine-containing drugs and pharmaceutical preparations has been studied. The reason for the failure of the colour reaction to obey Beer's law is discussed.

IN a study of the stability of thyroxine and of 3,5,3′-triiodothyronine (liothyronine) in alkaline solution, Maclagan, Bowden and Wilkinson (1957) and Lein and Michel (1959) have shown that these compounds break down with release of inorganic iodide. In an attempt to study these observations quantitatively, an assay for small amounts of inorganic iodide in the presence of iodothyronines and of iodotyrosines was required. The methods already described for the spectrophotometric and photocolorimetric micro-estimation of iodide (Zak, 1958; Armstrong, Gill and Rolf, 1961; Barker, 1962) are inapplicable in the presence of organically-bound iodine. Thus the ceric arsenite (Sandell-Kolthoff) method is not specific for iodide, since iodotyrosines and iodothyronines also catalyse the reduction of ceric ions by arsenious acid. Similarly in the oxidation of iodide to iodate with bromine or with permanganate, followed by determination of iodine liberated by potassium iodide, there is the risk of simultaneous oxidation of bound iodine, resulting in high values.

The iodate-starch method of Lambert (1951) is not applicable in the presence of compounds such as thyroxine, triiodothyronine or iodophthalein, since these alter the pH of the reaction mixture and thereby decrease the rate of formation of the blue colour. It has been found, however, that provided the pH is carefully controlled, treatment of an aqueous solution of iodide with a mixed potassium iodate-starch solution produces a colour which reaches a maximum value after about 12 min. and thereafter remains stable for at least a further 15 min. The method is applicable to the determination of not less than 4 p.p.m. of iodide in solution, for which a 10 ml. sample is required, or of about 0·004 per cent of iodide in an organic iodine-containing compound, assuming that 1 g. of material is examined.

EXPERIMENTAL

Preliminary Experiments

Preliminary experiments were made by adding to aliquots of an aqueous potassium iodide solution small volumes of the 1:2:1 potassium iodate-starch-N sulphuric acid spray reagent devised by Roche, Jutisz, Lissitsky and Michel (1950) for the detection of iodide on paper chromatograms. The colours obtained were unstable, the rate of fading depending on the acid content of the solution. Excess of iodate or of starch did not affect the colour. Using the extinction at 615 mμ, it was found that colour stability and the times at which maximum colour intensities are attained, are governed principally by the pH of the test solution before the iodate-starch reagent is added. Best results were obtained with a potassium hydrogen phthalate-hydrochloric acid buffer pH 2·2 (Bower and Bates, 1955) to which 0·5 per cent w/v of sodium sulphate was added to promote colour stability. This buffer solution gave excellent results for neutral or near-neutral solutions of iodide, but could not be used when the iodide solution contained sodium salts of organic acids, such as thyroxine, triiodothyronine and iodoxyl, since these substances altered the pH of the iodide-buffer solution. In these cases it was necessary to adjust the pH with a mineral acid, and to filter off any precipitated matter.

To extend the method to a range of organic iodine compounds, three modifications (A, B and C) have been used appropriately. In all instances there is a linear relationship between iodide content and extinction although the calibration line does not pass through the origin. The threshold value (i.e. iodide content for zero extinction) is about 120 μg./25 ml. When smaller amounts of iodide are to be determined, a standard amount of iodide must be added to overcome the "threshold".

Reagents and Procedures

All additions of iodide solution, buffer, 0·2N sulphuric acid, water and of iodate-starch reagent to be made accurately with burettes.

METHOD A. (Used for determination of inorganic iodide in neutral or near-neutral solution in absence of organically-bound iodine).

Potassium iodide solution containing about 30 mg. of potassium iodide, Analar, accurately weighed, in 1 litre of water.

Potassium hydrogen phthalate-sodium sulphate solution. Dissolve potassium hydrogen phthalate Analar (20·42 g.), sodium sulphate (anhydrous) Analar (10 g.) and chlorocresol B.P. (0·2 g.) in water, and dilute to 1 litre. (This solution is usable for several months.)

Buffer solution. Mix equal volumes of potassium hydrogen phthalate-sodium sulphate solution and of 0·1N hydrochloric acid. This buffer solution must be freshly prepared, as phthalic acid crystallises out on standing.

Iodate-starch solution. Mix 2 volumes of freshly-prepared 1 per cent starch solution with 3 volumes of 1 per cent potassium iodate (Analar) solution. This solution remains usable for one week.

Procedure. Dilute the test-solution, which should contain 150 to 500 μg. of iodide, to 15 ml. with water. Similarly dilute 7·5 ml. and 12·5 ml.

of potassium iodide solution (0·003 per cent) to 15 ml. with water (solutions used for calibration purposes). To all solutions add buffer solution (6 ml.), iodate-starch solution (2 ml.) and dilute to 25 ml. with water. Allow to stand for 15 min., then determine the extinction at 615 mμ in 1 cm. cells using a similarly prepared blank as reference liquid.

The iodide content (i) in μg. of the test solution is calculated from the equation

$$i = i_1 + (e_t - e_1)(i_2 - i_1)/(e_2 - e_1)$$

where e_t = extinction of test-solution; e_1 and e_2 = extinction of standard solutions (7·5 ml. and 12·5 ml. respectively); i_1 and i_2 = μg. of iodide contained in 7·5 ml. and 12·5 ml. of potassium iodide solution, respectively.

METHOD B. (Used for determining inorganic iodide in organic iodo-compounds, where the inorganic iodide content of the sample is not less than 350 μg.).

Potassium iodide solution containing about 60 mg. of potassium iodide, Analar, accurately weighed, in 1 litre of water.

Procedure. Transfer a suitable quantity of iodo-compound (*cf.* Table IV), accurately weighed, to a 30 ml. beaker and add water (10 ml.). Adjust potentiometrically to pH 2·2 with 0·2N sulphuric acid. Note the volume of acid required (*a* ml.). Rinse the electrodes with 5 ml. of water, add the washings to the contents of the beaker and allow to stand for 10–20 min. Filter through a No. 1 paper* and wash the beaker and filter with 17–*a* ml. of water, so that the volume of filtrate is 32 ml.

Determine the volume of 0·2N sulphuric acid required to adjust 10 ml. of potassium iodide solution (0·006 per cent) to pH 2·2 (about 0·6 ml.). Let this be *b*. Transfer 7·5 ml. and 12·5 ml. of potassium iodide solution to 50 ml. graduated flasks, then add to each flask *b* ml. of 0·2N sulphuric acid and water to 32 ml.

To all solutions add 5 ml. of iodate-starch reagent and dilute to 50 ml. with water. Allow to stand for 15 min., then determine the extinctions at 615 mμ in 1 cm. cells. The percentage of inorganic iodide (i) in the iodo-compound is calculated thus:

$$i = \frac{1}{w.10^4}[i_1 + (e_t - e_1)(i_2 - i_1)/(e_2 - e_1)]$$

where w = weight (or volume) of iodo-compound taken, in g. (or ml.).

METHOD C. (Used where inorganic iodide content is less than 350 μg.)

Procedure. Process as for Method B, but extract the iodide with potassium iodide solution (0·006 per cent, 10 ml.) in place of 10 ml. water. The percentage of inorganic iodide (i) in the iodo-compound is calculated thus:

$$i = \frac{1}{w.10^4}[i_1 - i_3 + (e_t - e_1)(i_2 - i_1)/(e_2 - e_1)]$$

where i_3 = μg. of iodide contained in 10 ml. of potassium iodide solution.

* Iodoxyl is soluble at pH 2·2 and does not require to be filtered.

Results and Discussion

The results of recovery experiments in which small amounts of potassium iodide were added to recrystallised *m*-iodobenzoic acid are shown in Table I, while the effects of various ions (generally in 10–100 molar excess) on the colour reaction are shown in Table II. Tables III and IV respectively include results, illustrating the use of the method for assay of small samples of pharmaceutical preparations and for estimation of iodide, when present as an impurity.

TABLE I

RECOVERY OF SMALL AMOUNTS OF POTASSIUM IODIDE IN THE PRESENCE OF *m*-IODOBENZOIC ACID USING METHOD C

Each 10 ml. aliquot of test-solution contained 100 mg. of *m*-iodobenzoic acid (recryst.) and 0·6 mg. of potassium iodide.

Potassium iodide added (expressed as iodide ion), µg.	Iodide recovered, per cent
20	108·5
40	100·5
60	101·6
80	101·9
100	101·8
120	102·1
140	98·95

The results for the inorganic iodide content of Thyroxine Sodium B.P. samples 1 and 2 are of interest. In the first place methods B and C gave closely corresponding results. Secondly the relatively high inorganic iodide content found in these samples is in agreement with a report that inorganic iodide detected during paper-chromatography of thyroxine and related compounds may derive from iodide present in the starting-material,

TABLE II

EFFECT OF SOME INORGANIC COMPOUNDS AND OTHER SUBSTANCES ON THE PHOTOCOLORIMETRIC DETERMINATION OF IODIDE BY THE IODATE-STARCH REACTION

Potassium iodide taken (as I)	Compound added	Amount added	Method	Iodide recovered, per cent
2 µM (254 µg.)	Na_2SO_4	200 µM (28·4 mg.)	A	101·9, 101·8, 101·9
” ” ”	NH_4Cl	” ” (10·7 mg.)	”	99·4, 100·4
” ” ”	NH_4NO_3	” ” (16·0 mg.)	”	101·3, 102·3
” ” ”	KCl	” ” (14·9 mg.)	”	99·3, 100·0
” ” ”	$KClO_3$	” ” (24·5 mg.)	”	99·0
” ” ”	KBr	20 µM (2·38 mg.)	”	94·2, 94·7
” ” ”	”	200 µM (23·8 mg.)	”	69·05
” ” ”	$KBrO_3$	” ” (33·4 mg.)	”	99·0, 99·3
” ” ”	Sodium acetate (anhyd.)	20 µM (1·64 mg.)	”	101·1, 99·9
” ” ”	” ” ”	200 µM (16·4 mg.)	”	50·3
” ” ”	Sodium citrate B.P.	20 ” (5·88 mg.)	”	91·9
” ” ”	” ” ”	200 ” (58·8 mg.)	”	Nil
” ” ”	Lactose	” ” (72·1 mg.)	”	99·6, 99·45
” ” ”	Ethanol	0·2 g.	”	99·9
” ” ”	”	2 g.	”	94·65, 94·55
4 µM (508 µg.)	Na_2SO_4	400 µM (56·8 mg.)	B	107·7, 107·5
” ” ”	KCl	” ” (29·8 mg.)	”	106·3, 106·4
” ” ”	KBr	40 ” (4·76 mg.)	”	84·35, 84·3
” ” ”	Iodine*	1·10 mg.	”	98·6
” ” ”	Ethanol	2 g.	”	88·7

* Solution acidified to pH 2·2 and extracted with chloroform.

rather than arise as a result of decomposition in the course of chromatography, as is sometimes stated in the literature (Donhoffer, Várnai, Szegvári, Farkas and Járai, 1960).

TABLE III

ASSAY OF SMALL AMOUNTS OF QUATERNARY AMMONIUM IODIDES BY IODATE-STARCH REACTION USING METHOD A

Compound	Theoretical iodine content, per cent	Loss on drying, per cent	Weight taken for determination, mg.	Recovery, calculated with reference to anhydrous material, per cent
Decamethonium iodide—				
Sample 1	49·56	7·29	0·5	100·0, 99·6
Sample 2	49·56	6·38	0·5	99·8, 100·3
Hexamethonium iodide ..	55·63	0·43	0·5	101·3, 101·0
Dimethyltubocurarine iodide	28·00	5·42	1	99·3, 99·3

Of two samples of Diodone Injection examined, one complied with the B.P. limit test for inorganic iodides and one did not.

Elemental iodine interferes if present before the addition of the iodate-starch solution, but it may be removed conveniently by extraction with chloroform.

TABLE IV

INORGANIC IODIDE CONTENT OF SOME ORGANIC IODO-COMPOUNDS AND INJECTIONS

Test substance	Amount taken for determination	Method	Inorganic iodide content
			per cent w/w
Diiodohydroxyquinoline B.P.	0·2 g.	C	Nil
Ethyl iodophenylundecanoate B.P.C. ..	0·5 g.	C	Nil
Iodoform	0·5 g.	C	0·008, 0·005, 0·008
Iodophthalein	30 mg.	B	1·455, 1·47*
Iopanoic acid B.P.	0·5 g.	C	Nil
Liothyronine sodium B.P.—Sample 1 ..	0·2 g.	C	0·028, 0·026
Sample 2 ..	0·2 g.	C	Nil
Pheniodol B.P.	0·2 g.	C	0·020, 0·020
Propyliodone B.P.	0·2 g.	C	Nil
Thyroxine	0·2 g.	C	0·028, 0·031
Thyroxine sodium B.P.—Sample 1 ..	40 mg.	C	0·30, 0·31, 0·28
" " ..	0·2 g.	B	0·285, 0·295
Sample 2 ..	40 mg.	C	0·30
" " ..	0·2 g.	B	0·275, 0·28
Sample 3 ..	0·2 g.	C	0·039
			per cent w/v
Diodone injection B.P.—Sample 1.. ..	1 ml.	C	0·0063, 0·0064
Sample 2.. ..	1 ml.	C	0·0026, 0·0019
Iodoxyl injection B.P.	0·5 ml.	C	0·022, 0·022

* Inorganic iodide content in iodophthalein found by titration with potassium iodate (Lang's method) 1·40 per cent.

In only three instances was the method inapplicable; these were iodoacetic acid (the relatively strong acidity caused a lowering of pH), thyroid and thyroglobulin.

The failure of the calibration line to pass through the origin may be explained as follows: since the hydrogen ion and iodide ion concentrations of the reaction medium are relatively small, some of the iodine produced in the oxidation of iodide by iodate

$$5I^- + IO_3^- + 6H^+ = 3I_2 + 3H_2O \quad \quad (1)$$

will be hydrolysed until a small but definite concentration of hypoiodous acid is established

$$I_2 + H_2O = HOI + H^+ + I^- \quad .. \quad .. \quad (2)$$

(cf. Allen and Keefer, 1955). This hydrolysis will result in a "threshold" below which no iodine is formed. Support for this hypothesis is provided by the specific extinction coefficients for the iodide-iodate-starch reaction and the iodine-starch reaction. The latter reaction, which is conducted in the presence of a considerable excess of iodide ion (thus suppressing hydrolysis of iodine by mechanisms 1 and 2) has an extinction coefficient of about 1470 with respect to iodine (Ovenston and Rees, 1950). The specific extinction coefficient for the iodide-iodate-starch reaction, on the other hand, is appreciably lower (about 650 for 250 μg. of iodide made up to a final volume of 25 ml., and 800 for 500 μg. of iodide in a final volume of 25 ml.). In the absence of excess iodide the iodine-starch colour also fails to obey Beer's law, and exhibits a "threshold" (Müller and McKenna, 1936).

In a recent publication Lambert and Zitomer (1963) attribute the iodide-iodate-starch "threshold" to loss of iodine by deposition in the interior of the starch helices. This explanation, however, is open to the objection that the "threshold" would then vary with the starch content of the reaction mixture, whereas provided the starch is present in excess the actual concentration has no effect on the reaction.

Acknowledgement. I wish to thank Dr. G. E. Foster for his interest throughout this investigation.

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The paper was presented by THE AUTHOR.

THE ESTIMATION OF MORPHINE, CODEINE AND THEBAINE IN OPIUM AND IN POPPY LATEX BY PAPER CHROMATOGRAPHY

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The recommended method involves separation of the morphine from the mixed alkaloids in chloroform using 0·1N sodium hydroxide and separation of the codeine and thebaine by means of a buffer solution of pH 2·0. The two fractions are then chromatographed on paper and after suitable colour development the areas of the spots are measured planimetrically. Constant areas for the same quantities of alkaloid are obtained by ensuring that the initial spots of material applied to the starting line are of constant area and that the running conditions are rigidly controlled, especially the temperature. To overcome the variability due to differences between sheets of chromatographic paper the "4 point bioassay" technique is used (in which two dose levels of standard solution of alkaloids and two dose levels of the unknown fraction are applied for each assay). The coefficients of variation for individual assays are for morphine, 3 to 6 per cent; for thebaine 5 to 7 per cent. Replicate assays can readily be run on one sheet of paper resulting in increased accuracy. The method is particularly suitable for small quantities of raw material (100 to 200 mg.) and is two to three times quicker than other published methods.

During an investigation of the possible function of the alkaloids of *Papaver somniferum* L., a rapid assay suitable for the main alkaloids in small samples of fresh latex was required. Published methods (Pfeifer, 1958; Milos, 1960) were time consuming, and of poor reproducibility due to the difficulty in removing contaminants (cf. Pride and Stern, 1954). Attempts to isolate the alkaloids by paper chromatography were tedious and frequently required larger amounts of raw material than were available.

An adaptation of the method of Fairbairn and Suwal (1959) for *Conium* has been found to give consistent results and we believe it to be of more general applicability than the original method.

Experimental

Preliminary work showed that attention to the following points was necessary to ensure consistent results.

(i) *Running solvent.* A single phase system with fixed proportions of individual solvents, as recommended by Smith (1960) and Betts (1961), was used.

(ii) The paper must be washed with running solvent.

(iii) *Running conditions.* Rigid temperature control is essential to prevent elongation and overlapping of the spots. Optimum separation and shape of the spots are obtained at 18° ($\pm 1°$) and when the solvent front moves about 25 cm. in 18 hr.

Application of the alkaloids. We confirmed previous experience (Fairbairn and Suwal, 1959) that the spots of material applied to the starting line must be of constant area. Details of how this is achieved are incorporated in the method.

Area quantity curve. Over a range of quantities which produced areas between about 3 and 7 cm.^{2} the area was proportional to the log quantity of alkaloid applied. Fig. 1 shows two area/log quantity curves for morphine representing the highest and lowest of nine curves, each based on separate sheets of paper. This satisfactory area/log quantity relationship allowed us to use the "4 point bioassay" method (Fisher, Parsons and Morrison, 1948; Ohtsu and Mizuno, 1952; Fairbairn and Suwal, 1959).

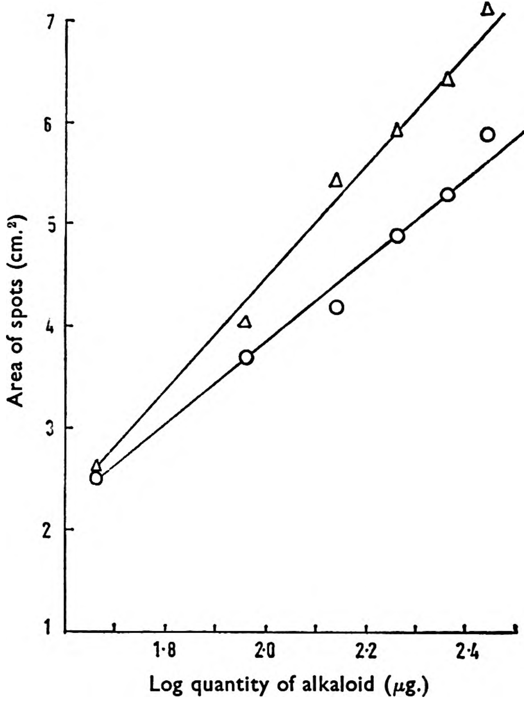

FIG. 1. Area/log quantity curves for morphine; each curve represents the results obtained on one sheet of paper. The two curves are the highest and lowest of nine curves, each from a separate sheet of paper, and thus indicate the range of the results for nine separate sheets of paper.

Recommended Method

Preliminary fractionation of the alkaloids from raw material is necessary for two reasons. Firstly the thebaine spot overlaps those of papaverine and the minor alkaloids. Secondly, the proportion of morphine is so much higher than that of the other alkaloids that the necessary quantity of extract cannot be applied to produce suitable spot areas for all the alkaloids simultaneously. Separation of the morphine is based on its solubility in 0·1N NaOH; traces of other phenolic alkaloids, such as

narceine and narcotoline, are removed in the later solvent extraction and paper chomatographic stages. Codeine and thebaine are separated from a chloroform solution of the remaining alkaloids by shaking with a buffer solution of pH 2·0; using artificial mixtures this gave 99 per cent recovery in contrast to only 75 per cent recovery when 0·2 per cent tartaric acid was used, as recommended by Pfeifer (1958).

Accurately weigh about 100 mg. of powdered opium and triturate with a few ml. of acid methanol (methanol 70 ml., hydrochloric acid B.P. 1 ml., water to 100 ml.). Filter and transfer to a 20 ml. volumetric flask; wash the filter with further solvent and make up to volume. For fresh latex, use 100 to 200 mg., add about 8 ml. of the acid methanol, stir, filter and make up to volume as above in a 10 ml. volumetric flask. Pipette 4·0 ml. of the acid methanol extract into a separating funnel containing 10 ml. 0·1N sodium hydroxide and shake for 1 min. Extract with 25, 15, and 10 ml. portions of chloroform, shaking for about 3 min. on each occasion, wash the combined chloroform layers with two 5 ml. portions of 0·1N sodium hydroxide and add these to the aqueous layer.

(i) To the *aqueous layer* add 0·5 ml. hydrochloric acid B.P. followed by 2 ml. solution of ammonia 10 per cent. Extract with 25, 20 and 15 ml. portions of ethyl acetate and wash the combined ethyl acetate layers with two 5 ml. portions of water. Reject the washings and evaporate the ethyl acetate extract to dryness on a water-bath. Dissolve the residue in methanol and make up to a volume of 1 ml. Use this solution, which contains the morphine, for the chromatographic procedure described below.

(ii) Extract the *chloroform layer*, with three 15 ml. portions of a buffer solution of pH 2·0 (M/5 KCl, 50 ml.; N/5 HCl, 10·6 ml., water to 200 ml.) shaking for about 5 min. on each occasion. Reject the chloroform layer, combine the acid layers and add 5 ml. N sodium hydroxide. Extract with 30, 25 and 15 ml. portions of chloroform and wash the combined chloroform layers with two 5 ml. portions of water. Reject the washings and evaporate the chloroforom extract to dryness on a water-bath. Dissolve the residue in methanol and make up to a volume of 1 ml. This solution contains the codeine and thebaine.

Standard solutions (*a*) morphine base: 1 per cent in methanol; (*b*) codeine base: 0·3 per cent; thebaine base: 0·2 per cent, in methanol.*

Use 0·01 ml. and 0·02 ml. of these solutions as standards.

Application of the solution to the paper. Sheets of Whatman No. 1 paper (46 × 54 cm.) washed with running solvent by descending technique for about 18 hr. and then dried at 100° are used with the direction of the fibres vertical.

With a cork borer of 7 mm. diameter make light impressions on the starting line, 3–4 cm. apart, thus ensuring uniformity of the spots. For each assay use 4 of these areas as follows; Standard S_1 (0·01 ml.); Unknown U_1 (0·01–0·05 ml. for morphine, double this volume for codeine and thebaine, so that the quantities in U_1 approximate those in S_1); Standard S_2 (0·02 ml.); Unknown U_2 (0·02–0·1 ml.). Apply the solutions

* As codeine and thebaine are estimated simultaneously, a *mixture* of the two alkaloids is used as a standard (Fairbairn and Suwal, 1959).

with an Agla syringe under constant drying conditions (Fairbairn and Suwal, 1959), adding sufficiently small quantities at a time to avoid exceeding the marked areas.

Development and Measurement of the Spot Areas

Running solvent. n-Butanol : acetic acid : water (5 : 1 : 2). Run the chromatogram in a tank maintained in a suitable water-bath at a temperature of 18° (±1°) for 18 hr. (±0·5 hr.). Dry the paper at 80° (10 min.) and spray with Dragendorff's reagent (Munier and Macheboeuf, 1951). Outline the spot areas in pencil while still wet and dry the papers in a current of warm air. Measure the outlined areas with a planimeter and calculate the amount of alkaloid from the following equation.

$$\log r = \frac{(U_2 + U_1) - (S_2 + S_1)}{(U_2 - U_1) + (S_2 - S_1)} \times \log 2.$$

Where r = ratio of quantities of alkaloid in Standard and Unknown spots.
S_1 and S_2 = spot areas corresponding to amounts of alkaloid in solutions of Standard applied to the paper.
U_1 and U_2 = spot areas corresponding to amounts of alkaloid in solutions of Unknown applied to the paper.

RESULTS

An artificial extract was prepared by dissolving known quantities of morphine, codeine, thebaine and papaverine in acid methanol. Papaverine was included to represent the alkaloids whose R_F values overlapped those of codeine and thebaine. Four or five separate assays, each involving three sets of area measurements on the same sheet of paper, were made by the recommended method. The results in Table I are typical of the variation of spot area within and between papers.

TABLE I

REPLICATE ASSAYS FOR MORPHINE IN ARTIFICIAL EXTRACT
(Actual amount present = 40·5 mg./20 ml.)

Chromatographic paper	Area of spots in cm.^{2}				Quantity of morphine (mg./20 ml.)
	S_1	S_2	U_1	U_2	
1	3·5	5·2	3·8	5·0	38·7
	3·6	5·0	3·7	5·0	38·8
	3·9	5·3	4·1	5·3	40·0
2	3·2	5·0	3·2	5·0	37·8
	3·4	5·4	3·5	5·4	38·5
	3·5	5·4	3·7	5·4	39·3

The results for morphine are: present, 40·5 mg., found (mean of 15 assays) 39·4 mg. s.d. 1·12; for codeine: present, 18·45 mg., found (mean of 15 assays) 18·24 mg. s.d. 0·55; thebaine: present, 8·93 mg., found (mean of 12 assays) 8·83 mg. s.d. 0·44.

Powdered opium. Three separate assays, each involving four sets of area measurements, were made on a sample of powdered opium. Recovery assays were also made by adding known quantities of alkaloid to the powdered opium and re-assaying. The results are shown in Table II.

TABLE II

SUMMARY OF RESULTS OF REPLICATE ASSAYS OF OPIUM AND OF OPIUM TO WHICH KNOWN QUANTITIES OF ALKALOID WERE ADDED. RESULTS EXPRESSED AS PER CENT ALKALOID

	Morphine	Codeine	Thebaine
Opium			
Mean of 13 assays	8·79	1·25	1·07
Standard deviation	0·53	0·076	0·073
Coefficient of variation	6·0 per cent	6·1 per cent	6·8 per cent
Opium + alkaloid 1			
Original + added amount	9·86	1·51	1·32
Amount found (mean of 4 assays)	10·37	1·54	1·31
Opium + alkaloid 2			
Original + added amount	19·50	2·57	2·30
Amount found (mean of 4 assays)	19·61	2·36	2·18

DISCUSSION

Reproducibility and Accuracy

The reproducibility of the method depends on producing constant areas of the running spot for identical quantities of the same alkaloid. To achieve this we have attempted to control every variable involved, but the differences between sheets of paper could not be eliminated. Table I shows the variation in spot area which is typical for a given quantity of morphine; for five sheets of paper it is 6 to 8 per cent of the mean. Fig. 1 represents the extreme variation for nine sheets of paper (8 to 10 per cent of the mean). This contrasts with our earlier experience with coniine (Fairbairn and Suwal, 1959) where a variation of up to 35 per cent of the mean using 4 sheets of paper occurred. This improvement may largely be due to the rigid temperature control. The results show that there still remains significant variation between different sheets of paper however, and by using the "4 point bioassay" technique a considerable increase in reproducibility may be obtained. For an artificial extract the standard error of the mean of four assays (P = 0·95) is for morphine and codeine ±3 per cent; for thebaine ±5 per cent. For powdered opium the corresponding figures for morphine and codeine are ±6 per cent and for thebaine ±7 per cent. The *accuracy* of the method is shown by the recovery experiments in Table II, and the 97–99 per cent recovery obtained with the artificial extract.

Since few previous workers quote adequate limits of error it is difficult to compare our method with theirs. However, Römisch (1961) who used a method involving elution of chromatographic spots followed by a spectrophotometric assay, quotes percentage errors for artificial extracts and for opium. Means of 3 assays of the artificial extract gave for morphine, errors of −5 to −8 per cent; for codeine 0 to −10 per cent and for thebaine −16 per cent. For opium (means of 2 assays) the errors were slightly greater ranging up to −17 per cent for thebaine.

Convenience

A certain amount of judgment is required in outlining the spots after spraying but results obtained by workers with no previous experience of the method show that the ability to do this consistently is rapidly acquired. The planimetric measurement of the final spot areas is the main time-consuming item; however we have found that, ignoring the overnight run in the chromatographic tank, the method is two to three times quicker than the other methods referred to earlier.

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The paper was presented by Miss Wassel.

IONISATION CONSTANTS OF SOME PENICILLINS AND OF THEIR ALKALINE AND PENICILLINASE HYDROLYSIS PRODUCTS

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Ionisation constants of several penicillins and of 6-aminopenicillanic acid in water at 25°, and approximate ionisation constants of the products of alkaline and penicillinase hydrolysis of some penicillins, are presented. The results suggest that mild alkaline hydrolysis of benzylpenicillin is not a simple reaction. The initial product (the penicilloic acid) has the same imino-group ionisation constant as the product of penicillinase hydrolysis, but this ionisation constant decreases after further treatment with alkali. The nature of this effect is discussed.

THE acid-base properties of penicillins and their degradation products, including the penicilloic acids, were of considerable importance in the determination of the structure of the penicillins (Chain, 1949), and are of value in the study of antibacterial activity and drug absorption. The accuracy of the ionisation constants determined earlier (Neuberger, 1949) was limited by the availability and purity of the penicillins, and there do not appear to have been any subsequent publications of more accurate results. Approximate constants of some penicilloic acids were determined (Neuberger, 1949) by back titration of penicillin which had been decomposed by alkali, and more accurate values were obtained for benzylpenicilloic acid by titration of an apparently pure sample of the monosodium salt (Mozingo and Folkers, 1949a). The former procedure involves the assumption that mild alkaline hydrolysis of a penicillin gives only the penicilloic acid. It is shown below that this assumption is of doubtful validity.

Subsequently, titration of the penicillinase hydrolysis product of benzylpenicillin (Benedict, Schmidt and Coghill, 1945) provided some of the evidence for the now accepted belief that the product of this reaction is the penicilloic acid (Pollock, 1960), although the ionisation constants reported by these authors were considerably different from those reported for monosodium benzylpenicilloate.

In this paper we report the ionisation constants of 6-aminopenicillanic acid and a number of penicillins, as well as the constants of the alkaline and penicillinase hydrolysis products of some of the latter.

EXPERIMENTAL

Materials

6 *Aminopenicillanic acid.* A recrystallised preparation of this material was supplied by Mr. F. R. Batchelor.

Penicillins. The penicillins, except ampicillin, were available as the sodium or potassium salt. Samples of benzyl penicillin, phenoxymethyl

penicillin and methicillin were normal commercial material. The other penicillins, which were purified by recrystallisation, were supplied by Dr. M. J. Soulal.

The purity of the penicillins, which were stored in a desiccator at 6°, was determined by an alkalimetric titration method which is routinely used in our laboratories, viz. the determination of the equivalents of alkali consumed by mild hydrolysis of the penicillin.

Penicilloic acids. Solutions of penicilloic acids were prepared by reacting a solution of about one millimole of the penicillin in about 40 ml. of water with 1 ml. of penicillinase solution. The penicillinase solution, supplied by Mr. F. R. Batchelor, was the cell supernatant from a culture of *Bacillus cereus* in a casein hydrolysate medium (Pollock, 1957). A pH of 5 to 7 was maintained by gradual addition of sodium hydroxide until the pH remained constant, the pH was then adjusted to 8·7, because this is the approximate equivalence point for the disodium salt of a penicilloic acid. The solutions were made up to 100 ml.

The total amount of alkali added in all the reactions was very close to 1 equivilant per mole of penicillin, indicating complete hydrolysis of the penicillin. Some of the solutions were tested for residual penicillin by the hydroxamic acid method, with negative results. Consequently, the concentration of penicilloic acid was calculated by assuming 100 per cent hydrolysis of the penicillin to the penicilloic acid.

Some penicilloic acid solutions were also prepared by alkaline hydrolysis of the penicillins. Details of these are given in Table IV.

Procedure

All solutions were made with water that had been double distilled in glass apparatus, boiled for 10–15 min. to remove carbon dioxide and stored in an effectively carbon dioxide-free atmosphere.

Solutions of the penicillins and 6-aminopenicillanic acid in water were titrated with 0·4M hydrochloric acid to pH 2·25 or a higher pH if precipitation of the penicillin free acid occurred. With some penicillins, particularly cloxacillin, a low concentration had to be used so that the titration could be continued to a reasonably low pH, thus enabling enough pK_a values for averaging to be calculated. The random scatter of the pK_a values obtained suggests that supersaturation effects can be neglected. Ampicillin and 6-aminopenicillanic acid, which are amino-acids, were also titrated with 0·3M sodium hydroxide, until 1 equivalent of the latter had been added.

The initial concentrations of the penicillins, corrected for purity, are given in Table I.

The solutions of penicilloic acids, of initial concentration about 0·01M, were titrated with 0·4M hydrochloric acid to pH 2·25.

Titrations

75 ml. of sample solution was titrated in a glass vessel of 200 ml. capacity, kept in a water-bath at 25 $\pm$ 0·1°. Titrant was added in 0·1 ml. portions from a 10 ml. burette calibrated in 0·02 ml. divisions.

The solution, through which nitrogen was bubbled, was stirred by a glass paddle-stirrer driven by an electric motor. The pH values were measured by a glass and saturated calomel electrode system, and a Pye "Dynacap" pH meter calibrated in 0·02 pH unit divisions and run from a constant voltage transformer (230 V). The meter was standardised immediately before and immediately after all titrations with buffer solutions made from Soloid tablets and kept at 25°. If a change of more than 0·02 pH unit was observed in a particular titration the latter was rejected. Standardisation was effected at pH 4 and 7 for titrations with hydrochloric acid and at pH 7 and 9·15 for those with sodium hydroxide.

Calculations

Acid ionisation constants, defined by equation (1), were calculated at each pH value measured in the buffer region of the group's dissociation.

$$K_a = \frac{(H)\,[A^-]}{[HA]} \quad \text{.. .. .. ..} \quad (1)$$

where (H) is the hydrogen ion activity. $[A^-]$ is the concentration of carboxylate ion or of un-ionised amino-compound, and [HA] is the concentration of un-ionised carboxylic acid or of the conjugate acid of the amino-compound, when K_a is the ionisation constant of a carboxy group or of an amino-(or imino-) group respectively. The constants so obtained are not thermodynamic constants since the definition of these involves the activities, instead of the concentrations, of all the species involved in the equilibrium. The constant defined by (1) has been called the "apparent" (Greenstein and Winity, 1961) or the "mixed" (Albert and Serjeant, 1962a) isonisation constant.

The constants were calculated from the relevant form of the Henderson (1908) equation; equation (2) for the titrations with HCl and equation (3) for the titrations with NaOH.

$$pK_a = pH - \log\left(\frac{c}{a - H} - 1\right) \quad \text{.. ..} \quad (2)$$

$$pK_a = pH + \log\left(\frac{c}{a} - 1\right) \quad \text{.. .. ..} \quad (3)$$

where c is the total sample concentration, a is the titrant concentration and H is the hydrogen ion concentration. Increase in the total volume of solution caused by addition of titrant was allowed for when the concentrations were calculated.

The hydrogen ion concentration in (2) can be obtained either from the measured pH, or from the titration of a blank solution which contains all components of the sample solution except the pure substance under investigation (Glasstone, 1942). As a result of the definition of pH, the former method gives the hydrogen ion activity and not the concentration, but the use of activity in place of concentration here is not infrequently found in the literature (Albert and Serjeant, 1962b). When the latter method is used, (2) becomes:

$$pK_a = pH - \left(\log \frac{c}{a - A_1} - 1\right) \quad \text{.. ..} \quad (4)$$

where A_1 is the concentration of acid added to the blank titration at the relevant pH. This procedure involves two assumptions; (*a*) that hydrogen ion concentrations are identical when the sample and blank solutions are at the same pH, i.e., that the hydrogen ion activity coefficients are identical when the two solutions are at the same pH, and (*b*) that the titrant is 100 per cent dissociated in the blank solution. The validity of these assumptions is theoretically dubious and there seems to be no need to use this method unless the sample solution contains titratable substances other than the sample itself.

The ionisation constants of the penicillins were calculated by both these methods. The results of the two calculations agreed to within 0·02 pK_a unit for all the penicillins thus showing that the above assumptions are acceptable under the conditions used in this work. This is important because the blank titration method had to be used for the penicilloic acid calculations, there being some titratable material in the penicillinase preparation.

Results

The penicillins (I) and penicilloic acids (II) have structures as follows:

```
              S                                      S
             / \                                    / \
RNHCH—CH   CMe2             RNHCH———————CH   CMe2
      |     |    |                   |           |      |
    //C——N——CHCOOH                COOH  HN———CHCOOH
   O
          (I)                                   (II)
```

Penicillins. Ionisation constants of seven penicillins and of 6-amino-penicillanic acid are given in Table I. Each result is the average of a set of pK_a values calculated at each pH measured during the titration. In all cases results from two titrations are given. The lowest number of values in a set was 7 and most sets contained more than 10 values. The mean value and the maximum deviation of each set of values is quoted.

Penicilloic acids. The penicilloic acids have three ionisation constants, two attributable to carboxy groups and one to the imino-group. The two carboxy group constants are evidently of similar magnitude and it was not possible to evaluate them from the experimental data of the present work.

Imino-group ionisation constants of four penicilloic acids, obtained by penicillinase hydrolysis of the penicillins are given in Table II. Again the results are the averages of several values and results from two titrations are given. However, the pK_a values from each titration showed a tendency to decrease as the pH decreased. An example is shown in Table III. Possible reasons for this decrease are suggested in the Discussion. The results in Table II were taken from the earlier part of the titrations, where the values generally showed less consistent downward variations. Thus each result is the average of 11–13 values, obtained over the pH range 6·5–5·0, and in each case 3–5 values obtained

TABLE I

IONISATION CONSTANTS OF PENICILLINS AND 6-AMINOPENICILLANIC ACID IN WATER AT 25°

Sample	R	Purity per cent	Initial Conc. (M)	pKa -COOH	pKa $-NH_2$
6 APA	H–	100	0·0081	2·30 ± 0·05 2·29 ± 0·03	4·90 ± 0·05 4·92 ± 0·03
Benzylpenicillin	$PhCH_2CO$–	99·3	0·0099 0·0093	2·73 ± 0·03 2·71 ± 0·05	
Phenoxymethyl-penicillin	$PhOCH_2CO$–	97·9	0·0098 0·0054	2·73 ± 0·05 2·74 ± 0·04	
Phenethicillin	PhOCH(Me)CO–	97·5	0·0099 0·0082	2·72 ± 0·02 2·74 ± 0·03	
Propicillin	PhOCH(Et)CO–	99·2	0·0048 0·0034	2·72 ± 0·03 2·72 ± 0·04	
Methicillin	2,6-(Me)₂-phenyl–CO– (ring structure: Me, CO–, Me)	97·5	0·0099 0·0096	2·76 ± 0·02 2·78 ± 0·03	
Cloxacillin	3-(2-Cl-phenyl)-5-Me-isoxazol-4-yl–CO– (ring structure: Cl, N, O, Me, CO–)	97·7	0·0025	2·73 ± 0·04 2·70 ± 0·03	
Ampicillin	$PhCH(NH_2)CO$–	99·3	0·0079	2·53 ± 0·04 2·52 ± 0·02	7·24 ± 0·02 7·25 ± 0·03

TABLE II

IMINO-GROUP IONISATION CONSTANTS OF SOME PENICILLOIC ACIDS IN WATER AT 25°

Penicilloic acid	pKa
Benzyl	5·31 ± 0·05 5·33 ± 0·04
Phenoxymethyl	5·18 ± 0·03 5·12 ± 0·04
Phenoxyethyl	5·31 ± 0·02 5·28 ± 0·04
Phenoxypropyl	5·30 ± 0·05 5·31 ± 0·05

over the pH range 5·0–4·4 were ignored in obtaining the average because of the downward trend.

Table IV presents imino-group ionisation constants of benzyl-penicilloic acid obtained by penicillinase and by alkaline hydrolysis of benzylpenicillin and titrated in 0·01M solution under various conditions. Here, because of the wide variations, the highest and lowest pKa values are quoted from a set of about 15 values calculated from single titrations. The values in each set decreased with decreasing pH.

Discussion

The ionisation constants obtained for the penicillins examined here are consistent with the approximate results reported by Neuberger (1949) for benzylpenicillin and some other natural penicillins, and the results for 6-aminopenicillanic acid are in agreement with the approximate results reported by Batchelor, Chain, Hardy, Mansford and Rolinson (1961) for this compound. The side chain has very little effect on the ionisation constant of the penicillin, except when a free amino-group is present as in 6-aminopenicillanic acid or ampicillin. In these cases the presence of the positively charged amino-group slightly increases the acid strength of the carboxy group. The small effect of the side chain on the ionisation constant is to be expected because of the large distance between the two groups.

TABLE III

Variation of pK_a of benzylpenicilloic acid with pH

pH	6·46	6·22	6·04	5·88	5·76	5·64
pK_a	5·38	5·37	5·35	5·34	5·34	5·33
pH contd.	5·54	5·43	5·33	5·23	5·12	5·01
pK_a	5·33	5·32	5·31	5·31	5·29	5·29
pH contd.	4·89	4·75	4·61	4·43		
pK_a	5·27	5·25	5·24	5·21		

TABLE IV

Imino-group ionisation constants of the product of hydrolysis of benzylpenicillin under various conditions

Conditions of hydrolysis	Initial NaCl* conc. (M)	pK_a
Penicillinase	None	5·34–5.20†
Penicillinase	0·016	5·24–5·13‡
Penicillinase; then NaOH added to give pH 12; 21 hr. at 25°	0·016	4·89–4·78
NaOH: pH 12 at 25° overnight	0·016	4·98–4·82
NaOH: pH 12 at 23° 4 hr.§	0·004	5·34–5·15
Penicillinase	1·6	4·81–4·70
Penicillinase; 6M HCHO	None	4·2‖

* NaCl was either added as such or produced by neutralisation of excess NaOH.
† 15 values. 11 values averaged to 5·31 ± 0·05 quoted in Table II.
‡ 15 values. 11 values averaged to 5·22 ± 0·04.
§ Solution maintained at pH 12 by gradual addition of NaOH for 1½ hr. till pH stopped falling, then kept without addition of NaOH for further 2½ hr.
‖ pH at half-neutralisation.

The results presented for the penicilloic acids are incomplete because of the absence of carboxy group ionisation constants. There are no reliable values of these constants in the literature; the only results to be found are a value of pK_a 2·95 (Neuberger, 1949) and one of pK_a 2·16 (Benedict, Schmidt and Coghill, 1945), both for benzylpenicilloic acid. The former value was obtained from a sample of monosodium benzylpenicilloate, but it is not clear whether the presence of a second carboxy group was allowed for in the calculation. The latter value was obtained from a penicillinase hydrolysate of benzylpenicillin. However, Benedict and others do not seem to have realised that there are two overlapping

carboxy dissociations, and they attributed the second ionisation constant that they determined from their titration, one of pK_a 4·7, to an acid group. This phrase has been interpreted (Henry and Housewright, 1947; Hamilton-Miller, Smith and Knox, 1963) to mean that a carboxy group of pK_a 4·7 is present in penicilloic acids.

The evidence presented by Neuberger (1949), based on comparisons with thiazolidines of various structures, provides reasonably conclusive proof that the ionisation constant of about pK_a 5 in penicilloic acids is due to the imino-group. The fact that titration in the presence of formaldehyde decreases the ionisation constants of amino- and imino-groups, but has little effect on carboxy group constants, has been used (Harris, 1923–24; Harris and Birch, 1930) in the allocation of ionisation constants of amino-acids. The result in Table IV shows that titration of benzylpenicilloic acid in the presence of formaldehyde decreases the pK_a 5·3 value to about pK_a 4·2, thus confirming that this constant is not primarily due to a carboxy group.

The imino pK_a value of 5·32 found (Table II) for the product of penicillinase hydrolysis of benzylpenicillin is in good agreement with the value of 5·25 reported by Mozingo and Folkers (1949a) for monosodium benzylpenicilloate, and is considerably different from the value of 4·7 reported for a penicillinase hydrolysate by Benedict and others (1945). The results presented here thus provide confirmation of one point in the surprisingly small amount of evidence (Pollock, 1960) that shows the product of penicillinase hydrolysis to be the penicilloic acid.

The only product of mild alkaline hydrolysis of a penicillin that has been reported (Mozingo and Folkers, 1949b) is the penicilloic acid. The results presented in Table IV show that the initial product obtained by hydrolysis at pH 12 for 4 hr. has approximately the same pK_a as penicilloic acid, but after 21 hr. at pH 12 a product with a lower pK_a is produced. Storage of a penicillinase hydrolysate at pH 12 for 21 hr. also gives a product of lower pK_a. The titrations done after storage at pH 12 for 21 hr. had higher initial sodium chloride contents than the other titrations, but this was shown not to be the cause of the lower pK_a's by titration of a fresh penicillinase hydrolysate in the presence of the relevant amount of sodium chloride (0·016M), which gave only a slight decrease of pK_a. Thus storage of benzylpenicilloic acid at pH 12 for 21 hr. has an effect which results in a considerable decrease of the pK_a of the imino-group. Since the basicity of the imino-group is unlikely to change as a result of conversion of the penicilloic acid to a different stereoisomer, the results imply that the acid is converted to some other compound. This conclusion is in agreement with observations of two spots on paper chromatograms of alkaline hydrolysates of some penicillins (Mansford, personal communication).

If this conversion occurs to some extent in penicillinase hydrolysates at pH 8·7, then the results in Table II–IV have been calculated from titrations of mixtures of penicilloic acid and the unknown compound. This could account for some of the decrease of pK_a observed in these titrations. However, an increase of 0·016M in initial sodium chloride

concentration decreases the pK_a by about 0·1 unit († and ‡, Table IV). The sodium chloride concentration increases by about 0·01M during titration over the pH range used for calculation of the pK_a values in Tables II–IV. Thus the salt effect associated with this increase probably accounts, at least in part, for the downward trend of pK_a values. Similarly, the low pK_a of 4·7 reported by Benedict and others (1945) could be attributed to a salt effect, because a pK_a of about 4·75 is obtained in the presence of 1·6M sodium chloride (Table IV).

The pK_a value of about 4·85 found after treatment with alkali for 21 hr. is close to the pK_a value of penilloic acids (Neuberger, 1949), which are the decarboxylation products of penicilloic acids. But decarboxylation at pH 12 would produce sodium carbonate, which would be titrated in the pH range 7·5–9. There was no evidence of this; thus titration of a penicillinase hydrolysate of benzylpenicillin in the presence of 0·5 mole sodium carbonate per mole penicillin required 0·5 ml. of titrant to change the pH from 9 to 6·7, while titration of the hydrolysate kept at pH 12 for 21 hr. required only 0·1 ml. of titrant for this pH change.

The formation of α-methyl penamaldate (III, R = Me) by the prolonged action of methanol on benzylpenicillin has been reported by Trenner (1949).

```
                                  HS
                                    \
PhCH2CONHC=========CH              CMe2
          |         |               |
         COOR      HN--------------CHCOOH
```

(III)

A tautomer of penamaldic acid (III, R = H) has been suggested (Goodall and Strafford, 1947) as a compound into which benzylpenicilloic acid might decompose before reacting with iodine. Its possible existence in equilibrium with benzylpenicilloic acid has been used (Cook, 1948) to explain the mutarotation of benzylpenicilloic acid in alkaline solution (Mozingo and Folkers, 1949b). The formation of penamaldic acid from benzylpenicilloic acid is consistent with the fact that thiazolidines generally exist in an equilibrium mixture of the ring form and an acyclic thiol form (Sprague and Land, 1957), although the presence of substituents generally stabilises the ring form. Some evidence for the existence of penamaldic acid in equilibrium with benzylpenicilloic acid was provided by the formation of penicillamine-cysteine disulphide on incubation of benzylpenicilloic acid with cystine at pH 7·5 and 37° (Levine, 1960).

The imino pK_a of penamaldic acid will differ from that of benzylpenicilloic acid as a result of (*a*) the presence of a double bond, which will decrease the pK_a, and (*b*) opening the thiazolidine ring, which will increase the pK_a. The effect of a double bond α- to a secondary nitrogen was studied by Starr, Bulbrook and Hixon (1932), using pyrrolidines and Δ^2-pyrrolines. They found that introduction of the double bond decreased the pK_a by 2·5–3·2 units. The magnitude of (*b*) can be

determined by reference to penicillamine, IV, which has an amino pK_a of 7·7 (Mozingo and Folkers, 1949c).

$$NH_2\text{—}CH(COOH)\text{—}C(Me_2)\text{—}SH$$

(IV)

Introduction of an *N*-methyl group slightly increases the pK_a of primary amines [e.g., methylamine pK_a 10·62, dimethylamine pK_a 10·77 (Albert and Serjeant, 1962c)] so that *N*-methyl penicillamine will have pK_a about 7·9. Ring closure of *N*-methyl penicillamine will give 3-carboxy-4,4-dimethylthiazolidine (V), which has an imino pK_a of 5·98 (Neuberger, 1949).

S; CH_2, CMe_2; HN——CHCOOH

(V)

Thus the net change of pK_a when the ring of a penicilloic acid is opened and a double bond introduced will be +1·9—(2·4 to 3·2), i.e.,—0·5 to 1·3. The observed effect when benzylpenicilloic acid is treated with alkali, a decrease of about 0·5 unit, is in reasonable agreement with the former value and penamaldic acid may well be a product of the action of alkali on benzylpenicilloic acid. Further work is in progress in an attempt to confirm this conclusion. If the conclusion is valid, it is clear from the results that the equilibrium between penamaldic acid and benzylpenicilloic acid is not rapidly established under the conditions of the titrations. This may be relevant to the large effect of experimental conditions on the iodine uptake of penicilloic acids (Weiss, 1959).

Less detailed experiments have indicated that alkali treatment of penicilloic acids other than benzyl has an effect similar to that discussed above.

Acknowledgements. The authors wish to thank Miss M. Zaleska for carrying out all the titrations, Mrs. J. Bellis for the penicillin purity determinations and Dr. J. H. C. Nayler for very valuable discussions.

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The paper was presented by DR. RAPSON.

A CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PHENOTHIAZINE

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A chromatographic assay of phenothiazine is described employing an acetonitrile-hexane partition system supported on Celite. Chromatographic separation is followed by ultra-violet measurement of the eluate fractions at 253 mμ. The system enables a determination of phenothiazine to be made in the presence of diphenylamine, carbazole, phenothiazone and phenothiazine sulphoxide, in addition to ingredients used as excipients in dispersible powders. The method has been tested on a range of samples and the results compared with those obtained by alternative procedures.

Although used mainly in veterinary medicine, phenothiazine is described in several national pharmacopoeias in most of which a Kjeldahl nitrogen determination is used as a method of assay.

Several more specific procedures have been proposed for example, the chromatographic assays of Gunew (1960) and Brierley and Langbridge (1961). For reasons described later, none of these can be considered entirely satisfactory for routine application, and an attempt has been made to devise a selective and accurate procedure suitable for routine use by a works analytical laboratory.

Experimental

Previous experience with partition chromatography on columns supported on Celite had shown that sharp and quantitative separation of structurally similar compounds can be achieved and led us to believe that this technique could be adapted for assaying phenothiazine. Of several solvent systems tried, the following was selected. Acetonitrile (1 vol.) was shaken with hexane (10 vols.), the lower layer was employed as stationary phase, and the upper layer was used to develop the chromatogram. The progress of the separation was followed by measuring the extinction at 253 mμ* of successive fractions of the column eluate. The relationship between extinction and the volume of eluate, using a repeatedly sublimed sample of phenothiazine, is illustrated in Fig. 1.

Method

Celite 545 prepared as described by Holbrook, Bailey and Bailey (1963) was used in a column 70 × 2·2 cm.

Preparation of sample and standard. Dissolve an accurately weighed quantity of about 100 mg. of pure phenothiazine in methanol and dilute to 100 ml. with methanol in a volumetric flask. Transfer a 10·0 ml.

* This represents the wavelength of maximum absorption of phenothiazine in the eluant phase.

aliquot of the above solution to a second 100 ml. volumetric flask and dilute to volume with methanol. Transfer 1·0 ml. of the latter solution (≡ 0·1 mg. phenothiazine) to a 50 ml. beaker, evaporate to dryness in a current of air, and set aside until required. Prepare an extract of the sample in a similar manner.

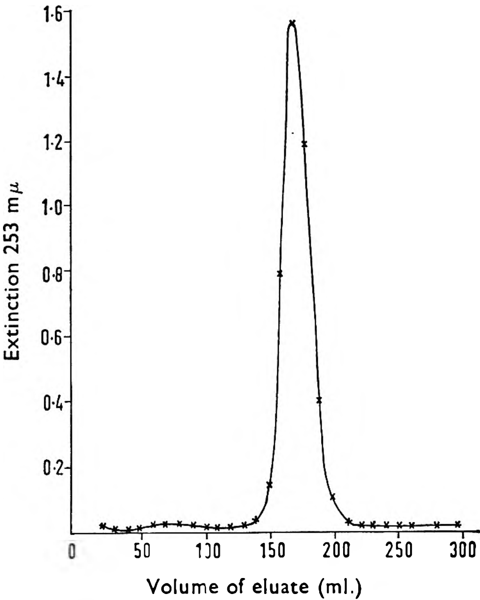

FIG. 1. Plot of extinction of eluate at 253 mμ against eluate volume.

Preparation of chromatographic column and subsequent treatment. Mix prepared Celite (25 g.) with stationary phase (12·5 ml.) in a 250 ml. beaker and transfer the mixture to the chromatographic column in portions of about 3 g., packing down firmly with a tamper between each successive addition. Dissolve the standard phenothiazine extract contained in the 50 ml. beaker in 1·0 ml. of stationary phase, add prepared Celite (2 g.), mix well and transfer quantitatively to the top of the stationary phase in the column. Carefully add eluent phase until the stationary phase is covered to a depth of about 40 cm. and adjust the flow of eluate from the column to about 5 ml./min. Maintain a constant flow rate by adding eluent phase to the top of the column and collect 35 successive 10 ml. fractions of eluate in 6″ × 1″ stoppered test tubes. Measure the extinction of each fraction in 1 cm. silica cells at 253 mμ against eluent phase in the reference cell. Repeat the entire chromatogram using the extract of the sample in place of the standard. Summate the extinction readings obtained from the chromatogram band of the standard and those of the corresponding band of the sample.

Then the percentage of phenothiazine in sample = $\frac{\epsilon_a \cdot W_s \cdot 100}{\epsilon_s \cdot W_a}$ where ϵ_a

and ϵ_s are the sums of extinction values under the sample and standard chromatogram peaks respectively. w_a = weight of sample (mg.) w_s = weight of standard phenothiazine (mg.)

Samples of likely impurities in crude phenothiazine were assayed as above. The curves relating volume of eluate to extinction at 253 mμ for phenothiazone, carbazole and diphenylamine, (Fig. 2) show that none of these compounds interferes with the determination of phenothiazine by the proposed method. Phenothiazine sulphoxide, remained on the column even after 400 ml. of eluate had been collected.

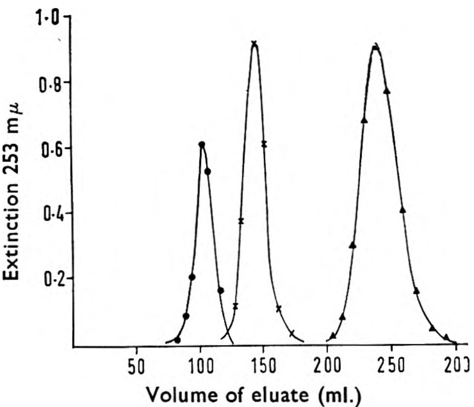

FIG. 2. Phenothiazine impurities. Plot of extinction at 253 mμ against eluate volume. ●——● Diphenylamine. ×——× Phenothiazone. ▲——▲ Carbazole.

RESULTS AND DISCUSSION

The high order of reproducibility by the proposed method is shown by the following results: pure phenothiazine 100, 99·9, 99·8 per cent: commercial sample I, 81·3, 80·6, 81·7 per cent: commercial sample II 88·2, 88·3, 87·8 per cent.

TABLE I

ANALYSIS OF ARTIFICIAL MIXTURE OF PHENOTHIAZINE AND IMPURITIES

Phenothiazine mg.	Phenothiazone mg.	Diphenylamine mg.	Carbazole mg.	Phenothiazine per cent added	Phenothiazine per cent recovered
9·04	1·41	1·17	1·15	70·7	69·8
9·32	8·34	8·46	8·69	26·8	27·4

Table I gives results on artificial mixtures of pure phenothiazine containing varying proportions of postulated impurities.

Of the alternative chromatographic methods, the Gunew procedure is extremely tedious to operate and in our opinion unsuitable for routine application, whilst in that of Brierley and Langbridge the phenothiazine passes through the column unabsorbed and is not separated from carbazole.

A comparison of the results obtained by the proposed method with those by the Kjeldahl nitrogen determination is given in Table II.

TABLE II

COMPARISON OF RESULTS BY PARTITION COLUMN CHROMATOGRAPHY WITH THOSE OBTAINED BY NITROGEN DETERMINATION

Sample	Nitrogen calc. to mol. wt. 199	Partition Chromatography
Phenothiazine pure		
1	100·0	100·0
2	99·3	98·8
Phenothiazine technical		
3	98·2	95·7
4	98·5	97·2
5	98·4	92·8
6	98·4	95·3
7	98·0	91·1 91·1
8	98·5	95·9
9	98·5	97·7
10	98·6	92·7
Phenothiazine dispersible powder		
11	93·0	92·5
12	93·8	89·9
13	93·9	87·0
14	98·0	85·5 86·0

The results on phenothiazine-dispersible powders from eight manufacturers fell within the range 81·7–92·5 per cent.

The Kjeldahl method gives results from 1–12 per cent higher than those obtained by the proposed method.

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The paper was presented by MR. HOLBROOK.

MULTIPLE SPOT PHENOMENA USING THIN LAYER CHROMATOGRAPHY OF PURE ORGANIC BASES

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Thin layer chromatographic behaviour of some sympathomimetic amines both alone and in the presence of acids in neutral and acidic solvent systems is reported. Thin layers of cellulose, with and without adhesive, alumina and silica gel were used; multiple spot formation only occurred with cellulose thin layers.

It has previously been shown that paper chromatography of amine salts (or of amines in the presence of an acid stronger than those of the solvent system) in neutral or acidic solvents may lead to two spots. One is of the amine in the same position as if chromatographed as the base, the other is present as a portion of the acid spot derived from the anion of the salt (Beckett, Beaven and Robinson, 1960).

The present investigation examines the possibility of multiple spot formation from a pure substance, using thin layer chromatography and several types of thin layers.

Experimental Methods

Materials. As described in the previous paper (Beckett, Beaven and Robinson, 1960) and also histamine phosphate having m.p. 128·5° [130°, after sintering 127° (Heilbron, Bunbury, 1943)]. Isoprenaline sulphate m.p. 128° [128° (Heilbron, Bunbury, 1943)]. Ephedrine sulphate m.p. 244° [245° decomp. (Heilbron, Bunbury, 1943)]. Ephedrine hydrochloride m.p. 219° [216°–220° (Heilbron, Bunbury, 1943)].

Solvent systems. (i) n-Butanol : acetic acid : water as 4 : 1 : 5 by volume. The organic layer was used. (ii) n-Butanol saturated with water.

Spray reagents. As described previously (Beckett, Beaven and Robinson, 1960). Ninhydrin 0·2 per cent w/v in n-butanol was used to detect histamine.

Preparation of plates. All plates used were 20 × 20 cm. They were dried at 100° for 2–3 hr., stored in a desiccator and reheated at 100° for 10 min. before use.

(i) *Cellulose.* (*a*) *With adhesive.* 20 g. of MN-cellulose powder 300 G (acc. Stahl) and 100 ml. of water for 5 plates. (*b*) *Without adhesive.* 20 g. of Whatman ashless cellulose powder mixed with 100 ml. of water for 3 plates.

(ii) *Silica gel.* 30 g. Kiesel-gel G-Merck (acc. Stahl) and 60 ml. of water for 5 plates.

(iii) *Alumina.* 30 g. Alumina oxydatum G-Merck (acc. Stahl) and 60 ml. water for 5 plates.

General Method

All experiments were made at least in triplicate to ensure that any slight possible differences in thickness of the layer were not giving misleading results. All results were within ±0·03 of the average figures recorded.

0·1 μmolar quantities were applied to the plates, 1·5 cm. from the bottom edge, as solutions of the pure base or salt in the presence of one and ten equivalents of the added acid.

All chromatograms were run for about 1½ hr., at room temperature, during which time the solvent front advanced 10 cm.

All the R_F values reported were calculated from the advanced edges of the spots.

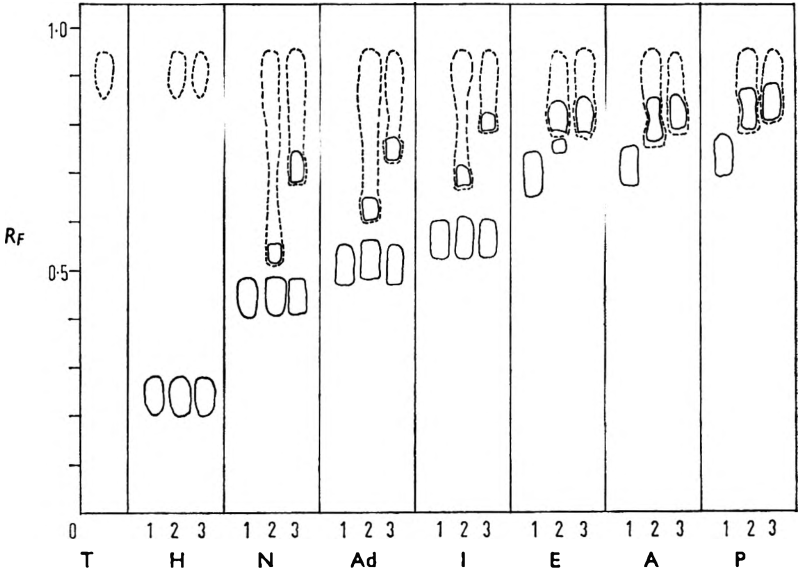

FIG. 1. Thin layer chromatography on cellulose of some sympathomimetic amines developed in the butanol: acetic acid: water solvent system when applied as 1 μl. of the pure base (or salts) or in solutions with one or ten equivalents of trichloroacetic acid. Continuous outlines indicate the amine, dotted outlines indicate the acid spot. 1. Pure base (0·05 M solution). 2. Pure base plus an equivalent amount of acid (0·05 M solution). 3. Amine (0·05 M solution) plus ten equivalents of acid. T, trichloroacetic acid. H, histamine. N, noradrenaline. Ad, adrenaline. I, isoprenaline. E, ephedrine. A, amphetamine. P, β-phenethylamine.

RESULTS

(i) *Cellulose Plates*

(a) *With adhesive.* The results using the n-butanol-acetic acid-water system and various amines of different R_F values in the absence and in the presence of 1- and 10-equivalents of trichloroacetic acid are shown in Fig. 1. The observed R_F values under these conditions are summarised in Table I.

TABLE I

R_F VALUES OF SOME SYMPATHOMIMETIC AMINES WHEN APPLIED TO CELLULOSE THIN LAYERS IN THE PRESENCE OF TRICHLOROACETIC ACID USING THE BUTANOL:ACETIC ACID:WATER SYSTEM

Amine	R_F	R_F of two amine spots (1-equivalent of acid present)		R_F of two amine spots 10-fold excess of acid present)	
Histamine	0·27	0·27	—	0·27	—
Noradrenaline	0·47	0·47	0·55	0·47	0·74
Adrenaline	0·55	0·56	0·65	0·55	0·77
Isoprenaline	0·60	0·61	0·71	0·60	0·82
Ephedrine	0·74	0·76	0·84	—	0·85
Amphetamine	0·75	—	0·85	—	0·86
β-Phenethylamine	0·77	—	0·87	—	0·88
Trichloroacetic acid	0·93	—	—	—	—

Similar experiments using dichloroacetic and picric acid as added acids yielded results shown in Fig. 2 and Fig. 3 respectively.

The results using the n-butanol:water saturated system and the above amines in the absence and in the presence of trichloroacetic acid are shown in Fig. 4 and for dichloroacetic and picric acid in Fig. 5 and Fig. 6 respectively.

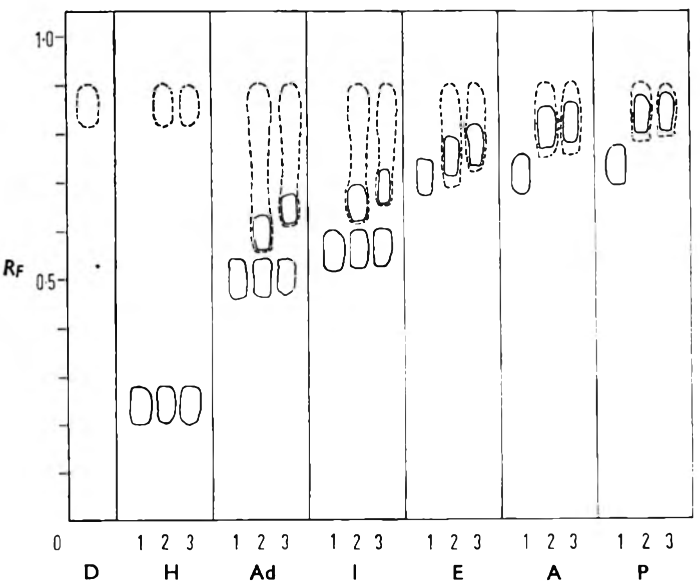

FIG. 2. As FIG. 1 but using dichloroacetic acid (D) instead of trichloroacetic acid.

(b) *Without adhesive.* The experiment was only carried out using the n-butanol: acetic acid: water system and a few amines in the absence and presence of 1- and 10-equivalents of trichloroacetic acid.

Double spot phenomena, similar to the above were obtained, but the R_F values were different, e.g. adrenaline alone had R_F 0·70 but gave two

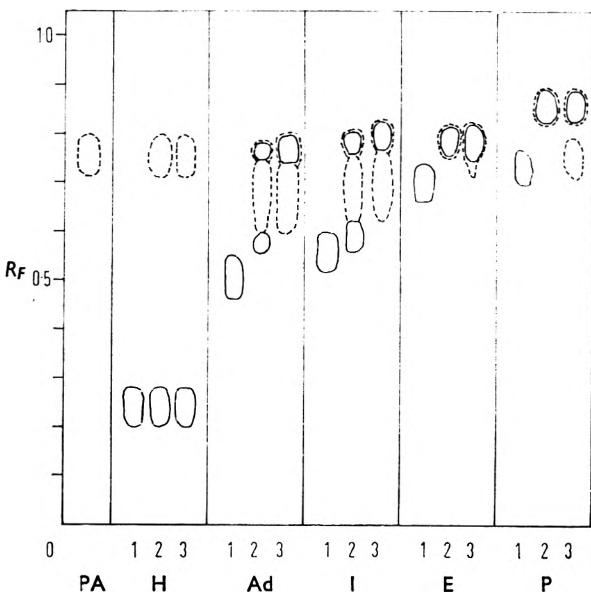

FIG. 3. As FIG. 1 but using picric acid (PA) instead of trichloroacetic acid.

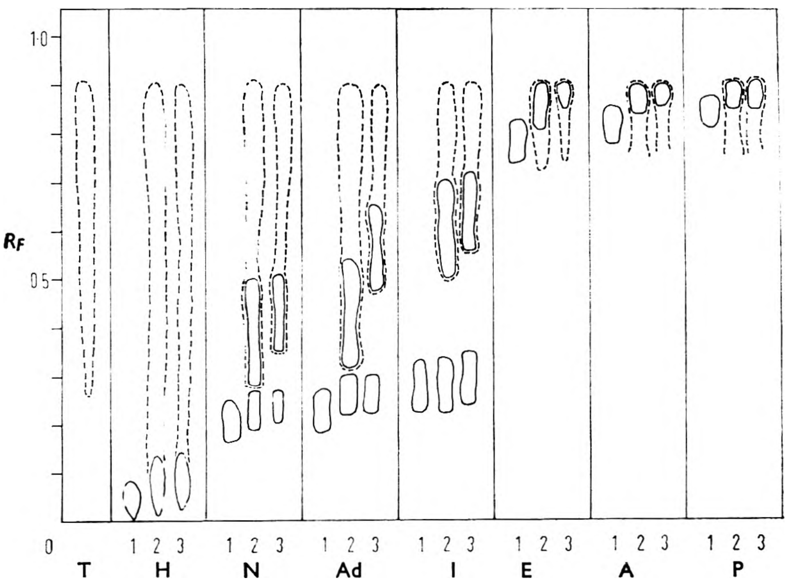

FIG. 4. As FIG 1 but using water-saturated butanol instead of the butanol : acetic acid : water system.

spots of adrenaline of R_F 0·70 and 0·83 in the presence of either 1- or 10-equivalents of trichloroacetic acid; isoprenaline alone had R_F 0·73 and gave two spots R_F 0·73 and 0·85 with 1- or 10-equivalents of trichloroacetic acid. Trichloroacetic acid alone gave a spot of R_F 0·95.

(ii) *Silica Gel Plates*

The results using the n-butanol: acetic acid: water system are shown in Fig. 7. Although the added trichloroacetic acid caused the amine spot to be elongated, two amine spots were not obtained.

In the butanol: water system the amine only moved slightly from the base line. The addition of 1- or 10-equivalents of trichloroacetic acid did not cause multiple spots of the amines.

(iii) *Alumina Plates*

The results using the n-butanol: acetic acid: water system are shown in Fig. 8; the addition of trichloroacetic acid did not cause multiple spot formation.

In the butanol-water system the amine spot remained in the base line.

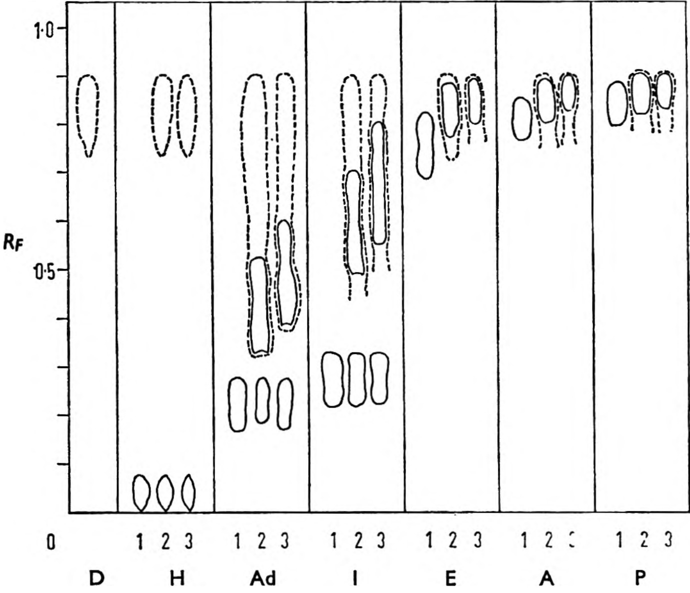

FIG. 5. As FIG 1 but using water-saturated butanol instead of the butanol: acetic acid: water system and dichloroacetic instead of trichloroacetic acid.

DISCUSSION

The results indicate that, just as in paper chromatography, it is possible to obtain two amine spots of an amine salt (or pure amine in the presence of one or more equivalents of acid) when a neutral or weakly acidic solvent system is used in thin layer chromatography involving cellulose as the thin layer.

This pattern of the multiple amine spots under the various conditions is similar to that observed in paper chromatography; detailed discussion is therefore unnecessary since the various factors leading to multiple spot formation in paper chromatography have been already presented (Beckett, Beaven and Robinson, 1960).

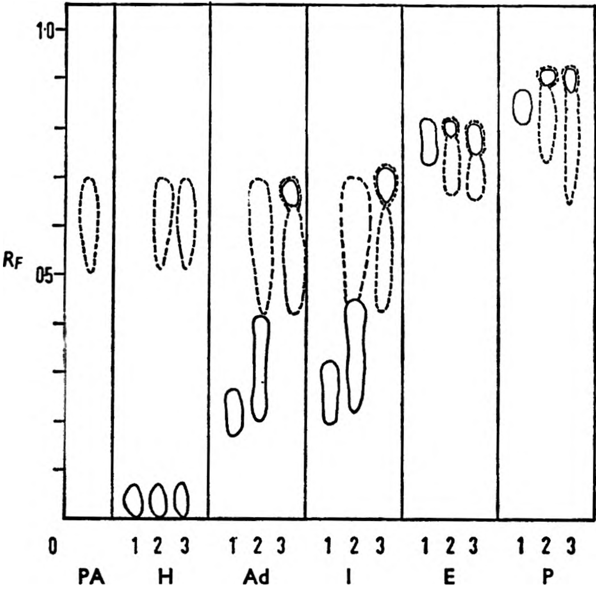

FIG. 6. As FIG. 1 but using water-saturated butanol instead of the butanol : acetic acid : water system and picric acid instead of trichloroacetic acid.

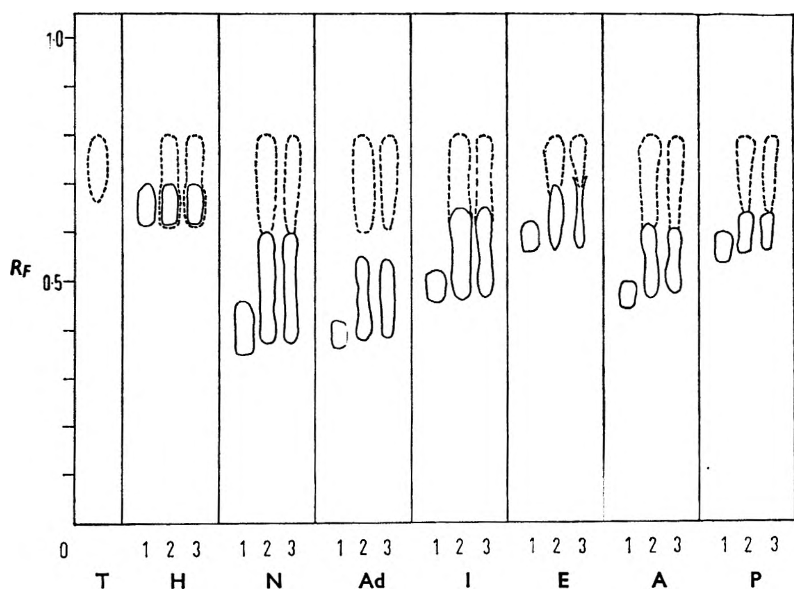

FIG. 7. Thin layer chromatography on silica gel of some sympathomimetic amines developed in butanol : acetic acid : water solvent system when applied as 1 μl. of the pure base (or salt) or in solutions with one or ten equivalents of trichloroacetic acid. Continuous outline indicates amine spot, dotted outline indicates the acid spot. 1. Pure base (0·05 M solution). 2. Pure base plus an equivalent amount of acid (0·05 M solutions). 3. Amine (0·05 M solution) plus a ten equivalent of acid.

The importance of the role of the added acid and the dependence of the formation of two spots of the amine upon the relative R_F of amine and added acid is depicted in Fig. 9 in which the importance of the equivalent concentration ratio of added acid to base may be seen by comparing Fig. 9 and Fig. 10.

Because a thin layer of cellulose powder without adhesive also gives multiple amine spots from a pure amine, the adhesive is not responsible for the phenomena.

The results, using a thin layer of silica gel and the butanol : acetic acid : water system, are completely different from the above in that only a single spot of an amine is obtained from a pure amine under conditions which would give two spots using cellulose (e.g. Fig. 7, Fig. 1). However the amine spots, using silica gel, are elongated by the presence of one or ten equivalents of added acid (Fig. 7). Since on silica gel, using a neutral solvent system, butanol : water, the amine spots and the acid spots move very little from the base line, no conclusion on the interaction of base with added acid can be made.

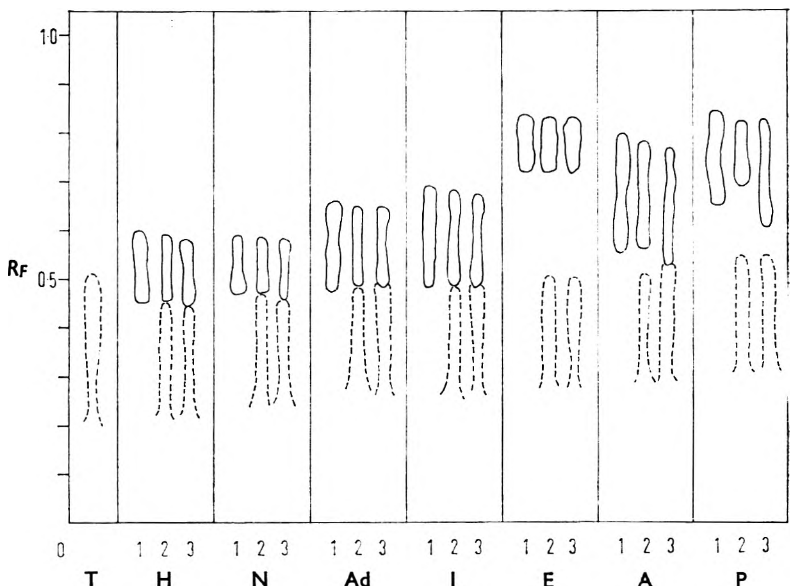

FIG. 8. Thin layer chromatography on alumina of some sympathomimetic amines developed in butanol : acetic acid : water solvent system when applied on 1 μl. of the. pure base or salt or in solutions with one or ten equivalents of trichloracetic acid Continuous outline indicates the amine, dotted outline indicates the acid spot. 1. Pure base (0·05 M solution). 2. Pure base plus an equivalent amount of acid (0·05 M solutions). 3. Amine (0·05 M solution) plus ten equivalents of acid.

With a thin layer of alumina and the butanol : acetic acid : water system, the amine spots are unaffected by the presence of one equivalent of added acid and only slightly affected by ten equivalents of acid (Fig. 8). Conclusions cannot be drawn using the butanol : water system, since amines and acids remained on the starting line.

In thin layer chromatography with alumina, adsorption forces operate exclusively; using silica gel, partition effect will in general be more important than adsorption forces; in a thin layer of cellulose, both partition and adsorption forces exist and their relative role depends upon the conditions.

Since added acid has a negligible effect on the running of amines on alumina, the effect of simple adsorption does not account for the multiple spots of amines on cellulose.

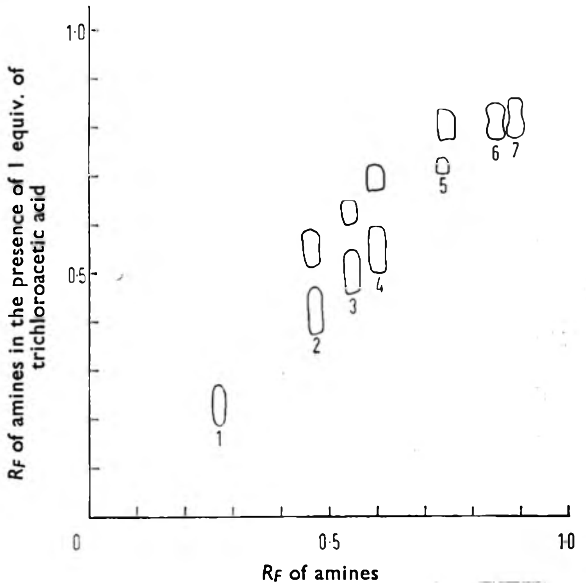

FIG. 9. Thin layer chromatography on cellulose of certain amines run in the butanol : acetic acid : water (4 : 1 : 5) solvent system when applied in the presence of 1 equiv. of trichloroacetic acid. 1. Histamine. 2. Noradrenaline. 3. Adrenaline. 4. Isoprenaline. 5. Ephedrine. 6. Amphetamine. 7. β-Phenethylamine.

On silica gel plates, the added acid, even in one equivalent ratio to the amine, has a pronounced effect on the running of the latter. It therefore seems probable that the added acid, as it moves along the plate, has a substantial effect on the partition of the amine between the running solvent and the stationary phase. However, two spots of the amine are not produced.

It is therefore suggested that the formation of two spots of amine, when an amine salt is subjected to thin layer chromatography on cellulose, results from the presence of the carboxy groups in the cellulose. The acid from the anion of the salt, in its movement along the plate, alters the partition of the amines between running solvent and stationary phase and the carboxyl groups make their contribution to the binding of amine (probably anion-cation association) in addition to the non-specific adsorption forces of the cellulose.

It seems that a continuity of adsorption forces along the direction of the running solvent is necessary for the multiple-spot phenomena of amine salts to occur, since two bands of amine do not result when the solutions and solvents are passed down in a column of cellulose under conditions which would give two spots using paper chromatography or thin layer chromatography on cellulose.*

The movement of the amines and acids down a column of non-bound cellulose is from one cellulose particle to another through a layer of solvent. With cellulose adhesively bound to glass in thin layers there is a continuity of adsorption forces.

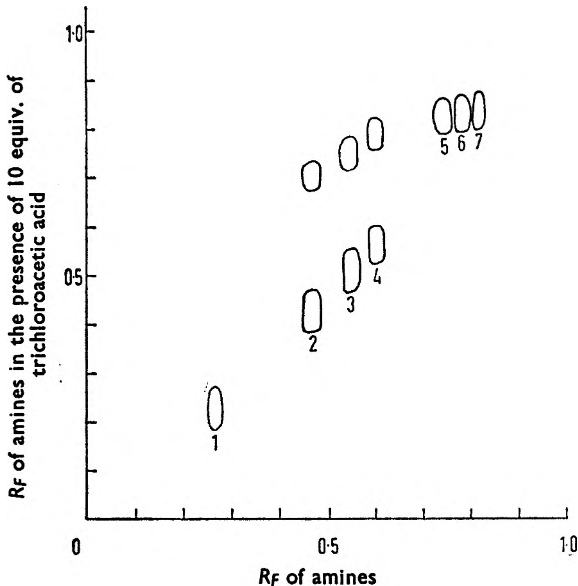

FIG. 10. Thin layer chromatography on cellulose of certain amines run in the butanol : acetic acid : water (4 : 1 : 5) solvent system when applied in the presence of 10 equiv. of trichloroacetic acid. Numbers as for Fig. 9.

The present investigation indicates that the presence of two amine spots in thin layer chromatography involving cellulose does not necessarily prove that two amines are present in the solution chromatographed. As in paper chromatography (Beckett, Beaven and Robinsin, 1960), especial care is therefore needed in interpreting information from thin layer cellulose chromatograms of amines in biological fluids or amines in solutions containing acids or salts.

Acknowledgement. One of us (N.H.C.) thanks the "Greek Foundation Scholarship" for a grant held while carrying out this research.

* Beckett, A. H. and Choulis, N. H. Adsorption forces in cellulose leading to multiple spot formation from pure amines using paper and thin layer chromatography. Paper to be presented at 23rd International Congress of Pharmaceutical Sciences, Munster, September, 1963.

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The paper was presented by MR. CHOULIS.

SPECTROSCOPIC STUDIES OF THE REACTION OF HYDROXYLATED PROMAZINES AND RELATED COMPOUNDS WITH SULPHURIC ACID

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The absorption spectra of the products from the reaction of various hydroxy- and methoxy-phenothiazine derivatives with sulphuric acid are described, and their application to problems of identification of metabolites of promazine and other phenothiazine drugs is discussed. A discussion of the nature of the reaction products is included.

Elucidation of the structure of the metabolites of phenothiazine drugs in man and animals has been complicated by the difficulty of synthesising suitable reference compounds, and by the lack of effective analytical methods for comparing unknown metabolites with standard compounds. It is further complicated by the small amount and impure condition of isolated metabolites.

In dog and man, promazine (I) is oxidised at the sulphur atom and demethylated at the tertiary nitrogen atom of the dimethylaminopropyl side chain (Walkenstein and Seifter, 1959).

$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$

(I)

R R′

(II)

In man the most important route of metabolism is by hydroxylation in the nucleus of the molecule and subsequent conjugation with glucuronic acid through the hydroxyl group introduced (Beckett and Bolt, unpublished results). No satisfactory method has yet been described for the determination of the position of hydroxylation and it has not yet been possible to synthesise all the ring hydroxylated promazines.

Phenothiazine derivatives react with concentrated sulphuric acid to give products with absorption patterns different from those of the original compounds in both the visible and ultra-violet regions of the spectrum. Dubost and Pascal (1953, 1955) showed that chlorpromazine and its sulphoxide give red colours with concentrated sulphuric acid and used the reaction in a colorimetric assay of these compounds. Rieder (1960) studied the products of this reaction from a number of phenothiazine derivatives and related the position of the main absorption band in the visible region of the spectrum to the nature of the substituent R in II. Street (1962) recorded the absorption maxima of a number of compounds of this type in both the ultra-violet and visible regions of the spectrum before and after treatment with sulphuric acid, and based a quantitative assay as well as a method of qualitative identification, on the intensity and position of the peaks in the region 270 to 300 mμ. Beckett, Beavan

and Robinson (1963) reported on the different absorption maxima in the visible region of the spectrum for the various derivatives in concentrated sulphuric acid solution, and used the extinction value for a semiquantitative assay in studies of the metabolism of chlorpromazine.

Because of the necessity to identify a promazine glucuronide of unknown structure, we have studied further the differences in spectral characteristics of various hydroxy-phenothiazines after treatment with sulphuric acid. It has been found that sufficient differences exist between the spectra produced from the reference compounds examined, to enable the position of ring hydroxylation of promazine metabolites to be determined.

Experimental

Materials

1-Hydroxypromazine hydrochloride (m.p. 210–211°), 4-hydroxypromazine (m.p. 166–167°), 3-methoxypromazine hydrochloride (m.p. 144–145°), and 3-hydroxyphenothiazine (m.p. 177·5–178·5°) were obtained from Dr. H. S. Posner; 2-hydroxypromazine (m.p. 176–178°) and 2-methoxyphenothiazine (m.p. 179–181°) from May and Baker Ltd.; 2-methoxypromazine maleate (m.p. 141–145°) from Smith, Kline and French Ltd.

Infra-red spectra of these compounds were consistent with their stated structures.

General Method of Reaction with Sulphuric Acid

The compound under examination (approx. 100 μg.) was dissolved in the minimum quantity of 70 per cent ethanol and added to 4 ml. of a solution of equal parts by volume of concentrated sulphuric acid and 70 per cent ethanol, to give a final concentration of approximately 25 μg./ml. The absorption measurements were made 15 min. after the phenothiazine compound was added to the ethanol-sulphuric acid mixture. Measurement in the visible region of the spectrum was made at the above concentration, but for ultra-violet measurements the solution was diluted with four times its own volume of the ethanol-sulphuric acid mixture.

Absorption Measurements

Absorption maxima were determined between 200–700 mμ for the untreated compounds and for the reaction products with a Beckman DK2 ratio recording spectrophotometer, using 1 cm. fused silica cells.

Results and Discussion

Discussion of the Method

The intensity of the colour produced in the reaction is largely dependent on the reaction conditions. The significant factors are the concentrations of the sulphuric acid and of the phenothiazine derivative, the temperature reached during the reaction, and the time taken for development of the colour. The influence of temperature increase resulting from mixing sulphuric acid and 70 per cent ethanol is minimised in the method described. Environmental temperature exerts a relatively slight effect on the extinction value. Under all conditions, extinction increases with time after mixing.

These factors have been evaluated in an attempt to devise a quantitative assay (unpublished results).

Using sulphuric acid concentrations between 25 and 65 per cent, v/v as in the method described above, reproducible absorption maxima can be obtained within a period of at least 1 hr. after mixing the reagents. Satisfactory extinctions (0·3–0·7) likewise result and allow the method to be applied to the qualitative identification of the phenothiazine derivatives examined. 70 per cent ethanol was used as a solvent because of its capacity to dissolve both the salts and the free bases of the compounds concerned.

Discussion of the Results

Although the absorption patterns of the pure phenothiazine derivatives examined are similar, sulphuric acid treatment produces sufficiently different spectra to allow the compounds to be distinguished. Fig. 1 shows the absorption spectra of the three hydroxypromazine derivatives examined and Fig. 2 their spectra after treatment.

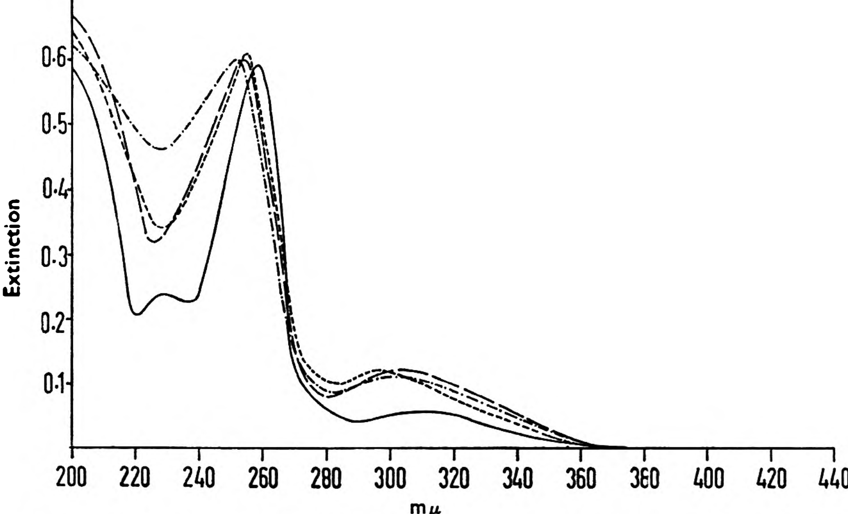

FIG. 1. Absorption spectra of some hydroxy- and methoxy-promazine reference compounds. —, 1-hydroxy-, — · —, 2-hydroxy-, — —, 3-methoxy-, - - - -, 4-hydroxypromazine.

In the case of 3-substituted derivatives (II), 3-hydroxypromazine is apparently not yet available, although the 3-methoxy compound has been synthesised (Posner, personal communication*). The absorption spectra of 2-methoxypromazine and 2-hydroxypromazine after sulphuric acid treatment are almost superimposable (see Table I); by analogy we suggest that the spectra from 3-methoxypromazine and 3-hydroxypromazine would likewise correspond, and that 3-methoxypromazine can therefore be used as a reference standard in determining the position of hydroxylation of promazine metabolites. The spectra of 3-methoxypromazine

* Since submission of the manuscript, 3-hydroxypromazine has become available.

before and after reaction with sulphuric acid are included in Figs. 1 and 2. Spectroscopic characteristics of equivalent sulphides and sulphoxides after treatment are identical.

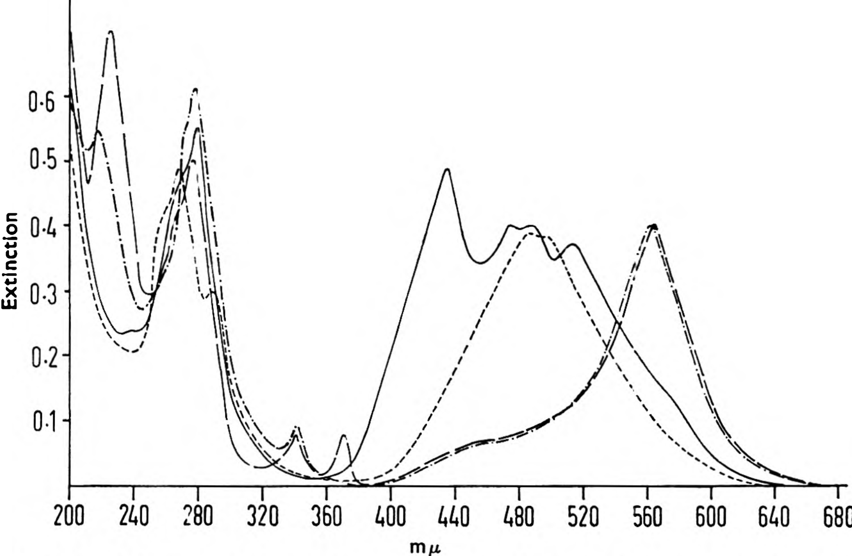

FIG. 2. Absorption spectra of the hydroxy- and methoxy-promazine derivatives of Fig. 1, after treatment with sulphuric acid. Key as in Fig. 1. Dilution (see method) is necessary at about 320 mμ.

The spectra of 2- and 3-substituted derivatives, after sulphuric acid treatment are similar, differing significantly only between 340 and 380 mμ. Further evidence that the peak at approx. 370 mμ is a feature of 3-substituted phenothiazines as opposed to their 2-substituted isomers is provided by the spectra generated from 2-methoxy and 3-hydroxyphenothiazine (Fig. 3). This Figure indicates that the spectral characteristics between 340 and 380 mμ after sulphuric acid treatment are controlled by the position of the ring substituent, and also shows that the nature of R′ in II has no qualititative effect on the spectrum in this region. Confirmation of this difference between 2- and 3-substituted derivatives is important since these are the most likely sites of biochemical hydroxylation.

TABLE I

ABSORPTION MAXIMA OF THE PRODUCTS OF THE REACTION BETWEEN VARIOUS HYDROXY AND METHOXY DERIVATIVES OF PROMAZINE AND 50 PER CENT V/V SULPHURIC ACID IN ETHANOL AT ROOM TEMPERATURE

Compound	λ (mμ)														
1-Hydroxypromazine			(270)	281						477		490		513	
2-Hydroxypromazine	219			278		343			(440)						558
2-Methoxypromazine	219			278		343			(440)						565
2-Methoxyphenothiazine	219			277		343		428							554
3-Methoxypromazine		225		279		342	372								565
3-Hydroxyphenothiazine		221		276		342	369		443						549
4-Hydroxypromazine			(262)	271	292						486		500		

Figures in brackets indicate shoulders on the main peaks.

The absorption maxima of the various compounds mentioned above are summarised in Table I, and a careful comparison of the spectrum of an unknown aglycone after sulphuric acid treatment with this reference data will provide conclusive evidence about the position of mono-hydroxylation of the promazine metabolite. It is also realised that evidence about the structures of aglycones from glucuronides of other phenothiazine drugs can be obtained in this way, provided it is possible to obtain the necessary reference data from the equivalent model metabolites.

It seems probable (see later) that, after sulphuric acid treatment, monoglucuronides of promazine would have the same spectra as their aglycones. Identification of metabolites would thus be facilitated because the glucuronides are more stable than their parent phenothiazine aglycones.

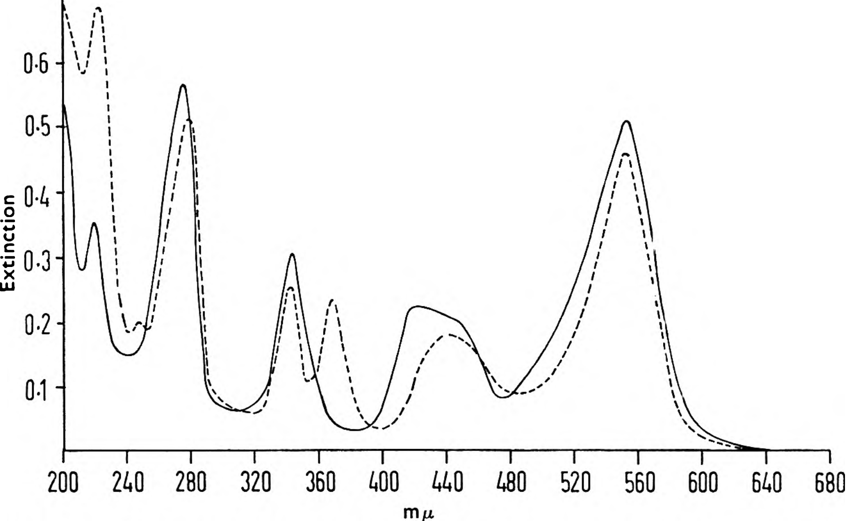

Fig. 3. Absorption spectra of the coloured oxidation products of 2-methoxyphenothiazine (—) and 3-hydroxyphenothiazine (- - -). Dilution as in Fig. 2.

The Nature of the Reaction Products

Reaction sequences have been described to explain the bivalent reversible oxidation of phenothiazine and related compounds (Cymerman Craig, 1960; Granick, Michaelis and Schubert, 1940; Michaelis and Granick, 1941; and Michaelis, Granick and Schubert, 1941). The oxidation involves semiquinone free radicals as intermediates.

Using chlorpromazine as an example, Borg and Cotzias (1962) have applied these theories to phenothiazine derivatives with a side chain on the nitrogen atom of the nucleus and have shown that the first product of oxidation arises by loss of one electron, and is a semiquinone radical ion. Its formula is best represented by III. This product then loses a second electron to form the phenazothionium ion IV. In the presence of water the phenazothionium ion forms a sulphonium base, and then chlorpromazine sulphoxide. The general process is represented as follows—(Borg and Cotzias, 1962).

$$\text{Sulphide} \underset{+e}{\overset{-e}{\rightleftharpoons}} \text{Semicuinone radical ion} \oplus \underset{+e}{\overset{-e}{\rightleftharpoons}} 2\oplus \text{ Phenazothionium ion} \xrightarrow[\text{slow}]{H_2O} \text{Sulphonium base } (+H^{\oplus})$$

$$\xrightarrow[\text{slow}]{H_2O} \text{sulphoxide } (+H^{\oplus})$$

$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$ (III) (Cl)

$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$ (IV) (Cl)

Our own unpublished studies with chlorpromazine have shown that the compound is oxidised to its colourless sulphoxide through a coloured intermediate by refluxing with 50 per cent sulphuric acid. Under mild conditions the reaction proceeds only as far as the coloured product. The absorption spectrum of this product is the same as that produced from chlorpromazine by Borg and Cotzias. Free radicals have been demonstrated in solutions of phenothiazine derivatives in strong sulphuric acid (Piette and Forrest, 1962). Hence sulphuric acid acts on chlorpromazine and other phenothiazine compounds under atmospheric conditions by an oxidation process involving bivalent loss of electrons. In the presence of the acid at room temperature the steps involving loss of protons in the above reaction sequence are obviously inhibited. The coloured product is the semiquinone radical ion.

TABLE II

ANODIC HALF-WAVE POTENTIALS OF OXIDATION OF SULPHIDES, AND ABSORPTION MAXIMA OF PHENAZOTHIONIUM IONS, OF COMPOUNDS RELATED TO PROMAZINE. ANODIC HALF-WAVE POTENTIALS ARE QUOTED FROM THE WORK OF EISDORFER AND HAINES (PERSONAL COMMUNICATION) AND ABSORPTION MAXIMA FROM THAT OF STREET (1962), AND RIEDER (1960)

Compound	R =	Anodic $E_{\frac{1}{2}}$	$\lambda_{max.}$ (mμ) of absorption of oxidation product
2-Methoxypromazine	OMe	+0·459	567
Promazine	H	+0·517	510–512
Chlorpromazine	Cl	+0·578	527–529
Fluopromazine	CF_3	+0·642	500

The ease of oxidative loss of electrons in II will be influenced by the character of R. Anodic half-wave potentials at pH 5 and 25° for loss of the first electron indicate that loss occurs more readily in the series R = OMe > H > Cl > CF_3 as one proceeds from groups which are electron donating to those which are electron attracting. The absorption maxima (Table II) of the sulphuric acid oxidation products however are not in the same sequence as above; wavelength is reduced in the order R=OMe > Cl > H > CF_3, since in the sulphide a +T effect is exerted by a -OMe substituent, and a -I effect is exerted by a -Cl or -CF_3 substituent,

whereas in the oxidation product the –Cl group exerts a +T effect while the effect of other substituents is unchanged. The +T effects from –OMe and –OH substituents are similar and the oxidation products have similar spectra. For this reason it is believed that glucuronides and aglycones would behave similarly.

$O^{\oplus}-C_6H_9O_6$

R R

$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$ (V) | $CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$ (VI) | $CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$ (VII)

In the product from oxidation with sulphuric acid a contribution is made by the phenazothionium ion. From 2-methoxypromazine, 2-hydroxypromazine and chlorpromazine this consists principally of structures IV and VI: from fluopromazine it consists of structures IV and V. As shown by the hydroxypromazines, the position of the hydroxyl group in the phenothiazine nucleus has an influence on the spectrum produced. 2- and 3-Substituted compounds give rise to phenazothionium ions which are *p*-quinones, whereas 1- and 4-substituted compounds give rise to *o*-quinones. In the latter the charge separation is less than in the former, and the wavelength of absorption (Table I) is thus lower. The ions from 1-hydroxypromazine and 3-hydroxypromazine have one end of the quinonoid system at the heterocyclic N atom, and those from 2-hydroxypromazine and 4-hydroxypromazine have the corresponding end of the system at the sulphur atom. Thus electronically the four phenazothionium ions are different, and this is illustrated spectroscopically by their absorption maxima (Table I), although in some of the cases the differences exist only in the fine structure of the spectrum.

Acknowledgements. We thank Dr. H. S. Posner of St. Elizabeth's Hospital, Washington, D.C., Messrs. May and Baker Ltd., and Smith Kline and French Ltd., for gifts of the compounds used in this study; also the Pharmaceutical Society of Great Britain for a grant to study the metabolism of phenothiazine drugs.

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The paper was presented by MR. CURRY.

ACTIVE SITES IN STEREOSELECTIVE ADSORBENTS AS MODELS OF DRUG RECEPTORS AND ENZYME ACTIVE SITES*

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The treatment of quinine-selective adsorbents by steam heat, in presence of water and by treatment with acid and dry organic solvents destroyed their selectivity whereas the adsorptive power of control adsorbents was almost unaffected by these treatments. A progressive increase in pH of the adsorbate solutions from pH 1 to 3 increased the adsorptive power of the selective adsorbents more than that of the control adsorbents. Increasing concentrations of sodium chloride in the adsorbate solutions increased the quinine-uptake on the adsorbents. Stereoselectivity is probably due to the formation of "footprints" in the surface of selective adsorbents. The similarities between the behaviour of active sites in biological surfaces and those in these stereoselective adsorbents are described.

The formation of adsorbents having stereoselective properties has already been described, e.g., for cinchona alkaloids (Beckett and Anderson, 1957). The use of stereoselective adsorbents in assigning configuration to morphine-type alkaloids has also been reported (Beckett and Anderson, 1959; 1960). Experiments are now outlined to ascertain how stereoselectivity is achieved; the data from these is used to indicate the similarities in behaviour of active sites in enzymes and drug receptors and the behaviour of those in stereoselective adsorbents.

Experimental

Preparation of the Adsorbents

This was carried out by the method of Beckett and Anderson (1960), with the modification of washing with distilled water after grinding the gel, to remove any residual sodium chloride before finally extracting with methanol.

Adsorption measurements. These were made by the method of Beckett and Anderson (1960) using quinine hydrochloride and 5-aminoacridine hydrochloride as adsorbates on control and quinine-selective adsorbents.

Treatment of Adsorbents

(*a*) *Treatment with Dry Organic Solvents*

An aliquot portion (10 g.) of the adsorbent (selective or control) was packed in a column provided with a dropping funnel and protected from moisture. Ether (2 ml.), acetone-ether 1 : 1 (10 ml.), ether (8 ml.) and ligroin b.p. 60–80° (10 ml.) were successively passed through the gel which was then transferred to a vacuum desiccator.

* Dedicated to Arne Tiselius in honour of his Sixtieth Birthday. This article was requested for *Perspectives in the Biochemistry of Large Molecules*, Academic Press, December, 1962, but failed to meet the dateline.

(*b*) *Heat Treatment*

Treatment by steam. Steam was passed for various periods of time through a flask containing an aliquot sample of the adsorbent. The adsorbent was filtered, extracted with methanol and dried at room temperature overnight.

Heating at 90°. The adsorbents (1 g. samples) were weighed in the flasks used to carry out the adsorption measurements and heated in an air oven for various periods of time.

Heating at 90° *in the presence of water.* The adsorbents were weighed as above, water (10 ml.) was added to the flasks which were shaken in an incubator at 90°, for various periods of time. The adsorbent was allowed to settle, as much as possible of the water was removed and the adsorption of quinine measured.

(*c*) *Acid Treatment*

Aliquot samples of the adsorbents were left in contact with 11·6N and 5·7N hydrochloric acid overnight, washed with distilled water till free from chloride, extracted with methanol and dried at room temperature overnight.

Other Treatments

Determination of "unextractable quinine" in selective adsorbents. The adsorbent (0·5 g.) was dissolved in 3N sodium hydroxide (5 ml.) and the solution was extracted with chloroform (10 g.). The quinine thus removed was then determined.

TABLE I

CONCENTRATION OF UNEXTRACTABLE QUININE AND THE ADSORPTIVE POWER OF SOME QUININE-SELECTIVE AND CONTROL ADSORBENTS

Adsorbent	Unextractable quinine expressed as moles × 10^6/kg. of adsorbent	Adsorption of quinine expressed as moles × 10^6/kg. of adsorbent
B_1	0·000	191
B_2	0·000	209
B_3	0·000	231
B_4	0·000	193
B_5	0·000	174
Q_1	232	328
Q_2	216	357
Q_3	154	324
Q_4	178	323
Q_5	314	320

Re-extraction of the stored adsorbents. The adsorbents, after storage at room temperature for the periods stated in Table II, were extracted with hot methanol for 5 hr. and the quinine uptake and "unextractable quinine" determined.

Effect of pH on selectivity. The pH was varied by carrying out the adsorption measurements in solutions of acetic acid of different concentrations.

Effect of strong electrolytes on adsorption. Solutions of various concentrations of sodium chloride in 0·5 per cent acetic acid were used.

Adsorption of quinine by control and selective adsorbents from these solutions was determined.

Determination of the loss of water of the adsorbents on heating. The adsorbent (1 g.) was heated at 200° to constant weight.

TABLE II

EFFECT OF RE-EXTRACTION WITH METHANOL OF STORED ADSORBENTS ON THE AMOUNT OF UNEXTRACTABLE QUININE AND ON THEIR ADSORPTIVE POWER

Adsorbent	Time of storage in months	Unextractable quinine expressed as moles × 10^5/kg. of adsorbent		Adsorption of quinine expressed as moles × 10^6/kg. of adsorbent	
		Before re-extraction	After re-extraction	Before re-extraction	After re-extraction
B_5	3	0·000	0·000	174	174
B_6	4	0·000	0·000	202	204
Q_6	4	314	202	323	343
Q_6	14	276	213	307	333
Q_7	10	264	173	333	345

RESULTS

Some representative results from many batches of adsorbents are presented herein. Batch to batch variation occurred in the absolute figures of adsorptive power, but the relative figures and the patterns described below did not. Subsequent unpublished work has shown that batch to batch variation may be almost eliminated by rigid temperature control during the drying of the gel to form the adsorbent.

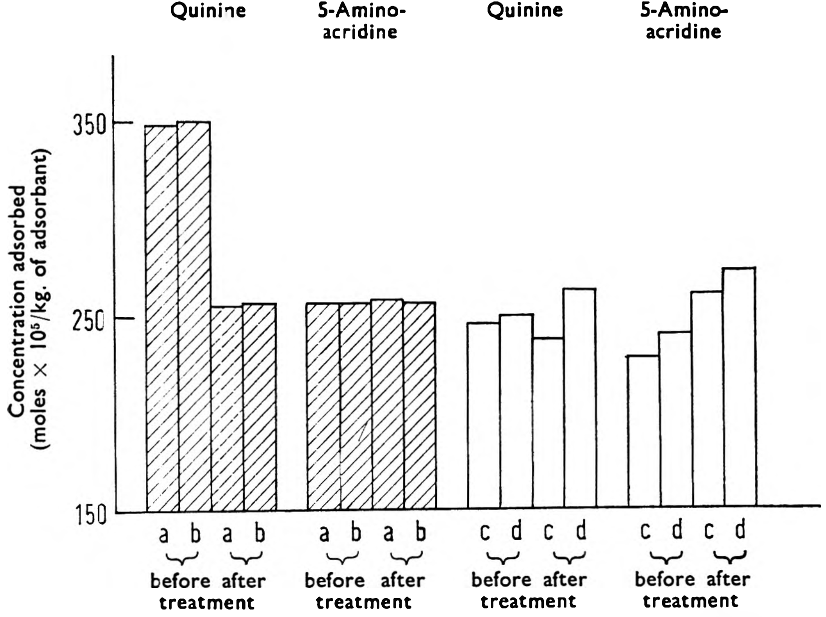


FIG. 1. Adsorption of quinine and 5-amino-acridine on quinine-selective and control adsorbents before and after treatment with dry organic solvents. ▨ Quinine-selective adsorbent; □ Control adsorbent. The letters indicate various batches.

The initial concentration for the quinine adsorption experiments was 35×10^{-5}M except for the measurements in the presence of sodium chloride in which the initial concentration was 50×10^{-5}M; for the 5-aminoacridine experiments, 40×10^{-5}M was used. All results quoted below are calculated with reference to the adsorbent dried to constant weight at 200°. Control adsorbents are indicated in the Tables by the letter B and quinine-selective adsorbents by Q; the other figures indicate various batches.

When the selective adsorbents were washed with dry organic solvents to eliminate free water (Trueblood and Malmberg, 1949), the adsorptive power for quinine was reduced to values comparable with those of the control adsorbents. This treatment did not release any of the unextractable quinine nor did it affect the mean adsorptive power of selective or control adsorbents for 5-aminoacridine (Fig. 1).

When steam was passed through the selective adsorbents their adsorptive power decreased markedly in less than 1 hr., while the same treatment increased, though to a lesser extent, the adsorptive power of the control, to give values approaching those of the selective adsorbents (Fig. 2).

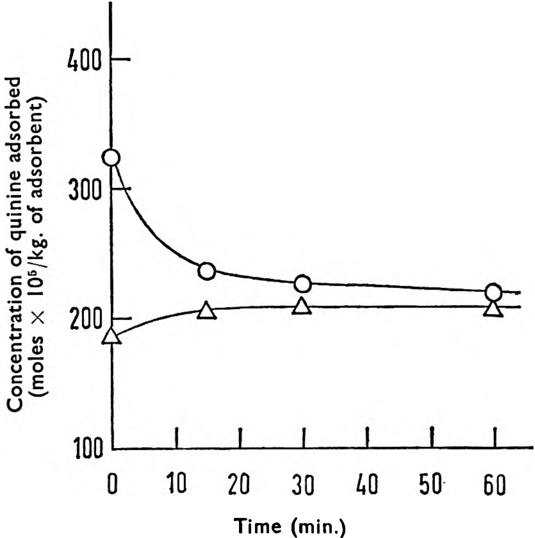


FIG. 2. Effect of steam treatment on selectivity. ○ Quinine-selective adsorbent. △ Control adsorbent.

Heating the adsorbents at 90° in aqueous suspension produced the same effect as steam treatment but at a slower rate, whereas dry heating at the same temperature for comparable times gave virtually no change in the adsorptive power of selective and control adsorbents (Fig. 3).

Acid treatment with 11·6N hydrochloric acid greatly reduced the adsorptive power of the selective adsorbent and *slightly* reduced that of

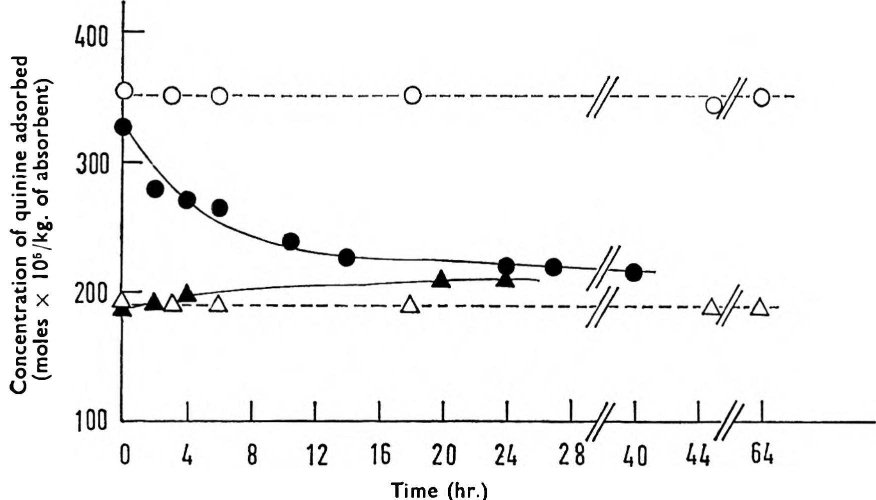


FIG. 3. Effect of heating at 90° on the adsorption on quinine-selective and control adsorbents. ● selective adsorbent heated in presence cf water. ▲ control adsorbent heated in presence of water. ○ selective adsorbent air heated. △ control adsorbent air heated.

the control so that both values became not too dissimilar; under comparable conditions 5·7N hydrochloric acid only partially reduced the adsorptive power of the selective adsorbent (Fig. 4).

Progressively increasing the pH of the solutions for the adsorption measurements (from pH 1 to 3), increased the adsorptive power of the

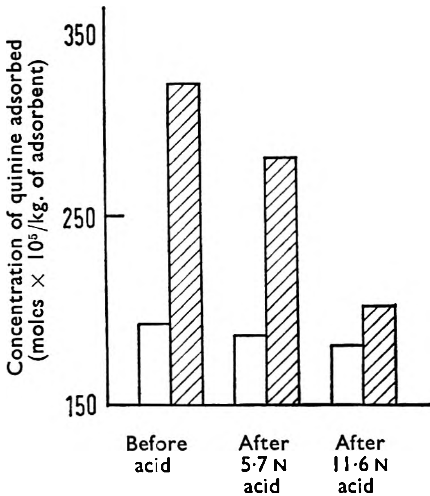


FIG. 4. Effect of treatment with 11·6N and 5·7N hydrochloric acid on selectivity. ▨ Quinine-selective adsorbent. □ Control adsorbent.

selective adsorbent and to a lesser extent that of the control so that selectivity was greater at the higher pH values (Fig. 5).

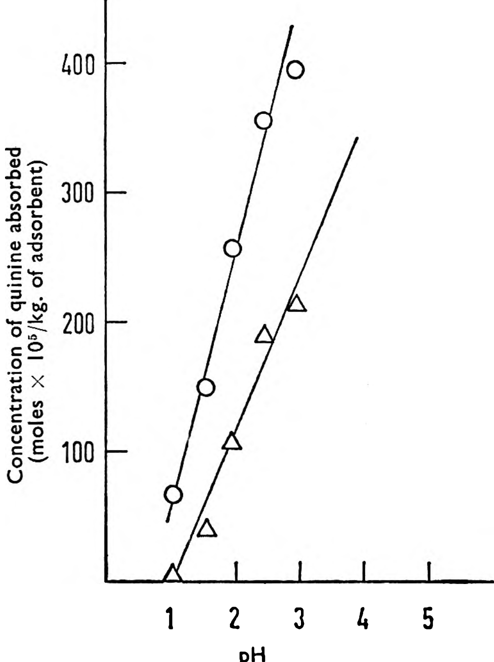


FIG. 5. Effect of pH on the adsorption of quinine on a selective and control adsorbent. ○ Quinine-selective adsorbent. △ control adsorbent.

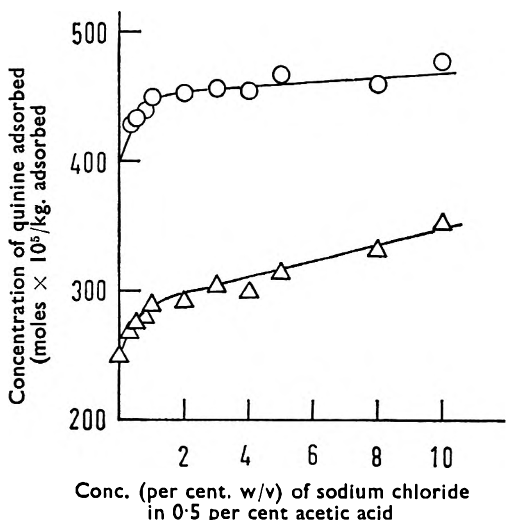


FIG. 6. Effect of sodium chloride on the adsorptive power of a quinine-selective and control adsorbent. ○ Quinine-selective adsorbent. △ Control adsorbent.

Increasing concentrations of sodium chloride up to 1 per cent in the solutions for adsorption measurements gave a significant increase in the adsorptive power of the selective adsorbent and to a slightly lesser extent of the control; beyond this concentration the increase of adsorptive power with concentration was less pronounced (Fig. 6).

The quantities of "unextractable quinine" in various batches of selective adsorbents are shown in Table I, together with the adsorptive power of the respective adsorbents.

Quinine was not liberated from the selective adsorbents when they were shaken with chloroform in the absence of sodium hydroxide.

Re-extraction of stored selective adsorbents reduced the amount of unextractable quinine and increased the adsorptive power of the adsorbent, while similar treatment of stored control adsorbents did not alter their adsorptive power (Table II).

The loss of water from some adsorbents on heating at 200° to constant weight is shown in Table III.

TABLE III

DETERMINATION OF THE LOSS OF WATER FROM QUININE-SELECTIVE AND CONTROL ADSORBENTS ON HEATING AT 200° TO CONSTANT WEIGHT

Control adsorbent	Per cent of loss of water (w/w)	Selective adsorbent	Per cent of loss of water (w/w)
B_1	15·5	Q_2	17·9
B_2	15·3	Q_4	19·4
B_3	18·0	Q_8	18·2
B_7	17·0	Q_9	19·9
B_8	16·9	Q_{10}	17·5
B_9	16·3	Q_{11}	20·2

DISCUSSION

Formation of Silica Gel

The formation of silica gel involves the elimination of water from orthosilicic acid to form spherical primary particles or micelles. As the gelation proceeds these micelles grow in size and condense together to give a very open but continuous structure extending throughout the medium and interpenetrated by the liquid phase. During drying, the

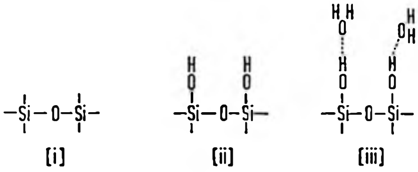


FIG. 7. Different types of chemical groups at the surface of silica gel. (i) siloxan group. (ii) silanol group. (iii) hydrated silanol group.

gel shrinks owing to the pressure exerted upon the structure by the surface tension of the liquid around the boundary of the gel. The packing density increases and the pore diameter diminishes; as certain particle to particle bonds break, portions of the network thus released come into contact with other members and new contacts and bonds are

formed. The shrinkage, essentially an irreversible process, proceeds until the mechanical strength of the gel can withstand the pressure of the surface tension (Iler, 1955). The rigid xerogel thus obtained consists of three dimensional silicon-oxygen lattice work (see Fig. 8). This aggregate structure accounts for the following characteristic features of silica gel; firstly it is highly porous due to the high specific surface area which can reach values of 1,000 m.^{2}/g. (Klein, 1961). Most of this area is present as internal surface in the form of pores of variable shapes and diameters. Secondly the surface-energy is heterogeneous owing to different types of chemical groups and their local geometry on the amorphous surface (Klein, 1962). Under normal conditions it is possible to distinguish siloxane groups (Fig. 7, i), silanol group (Fig. 7, ii) and hydrated silanol groups with physically bound or free water (Fig. 7, iii).

Theory of the Formation of "Active-site" Footprints

In selective adsorbents, the foreign organic molecules dissolved in the liquid phase will be attracted to the silanol surface as the structure of the gel is built up. Some of the molecules will be mechanically trapped within the primary particles as they grow in size: others will be persorbed in the fine pores and acted upon by two or more neighbouring surfaces at the same time. The remaining molecules will be adsorbed to the surface while the gel structure is still flexible. On drying and subsequent extraction with methanol, the adsorbed and some of the persorbed molecules will be removed leaving on the gel surface their footprints and partial footprints to become part of the rigid structure of the adsorbent. The molecules trapped within the silica framework will not be extracted by this treatment.

We conclude for reasons given below that stereoselectivity is due to the formation of these "active-site" footprints in the selective adsorbents. These footprints will preferentially adsorb the molecules used to make them, rather than other molecules. Alternative compounds of not too dissimilar structure, having "like" configuration will fit into these footprints better than will their isomers, although not quite so well as the reference molecule itself (cf. Beckett and Anderson, 1957, 1959, 1960).

Evidence Against Stereoselectivity Being Due to Foreign Molecules Trapped Inside the Selective Adsorbent

After the methanol extraction of the gel formed in the presence of quinine, there remains some "unextractable" quinine in the product. Instead of footprints accounting for selectivity, it has been suggested that these trapped molecules may attract the adsorbate molecules and may account for differences in the adsorptive powers of selective and control adsorbents (Morrison, Worsley, Shaw and Hodgson, 1959). Our case against this explanation of selectivity and against any explanation based on differences in surface area per g. of selective and control adsorbent rests on the following evidence:

1. The selective adsorbents are stereoselective to enantiomorphs and diastereoisomers (Beckett and Anderson, 1959 and 1960). Furthermore,

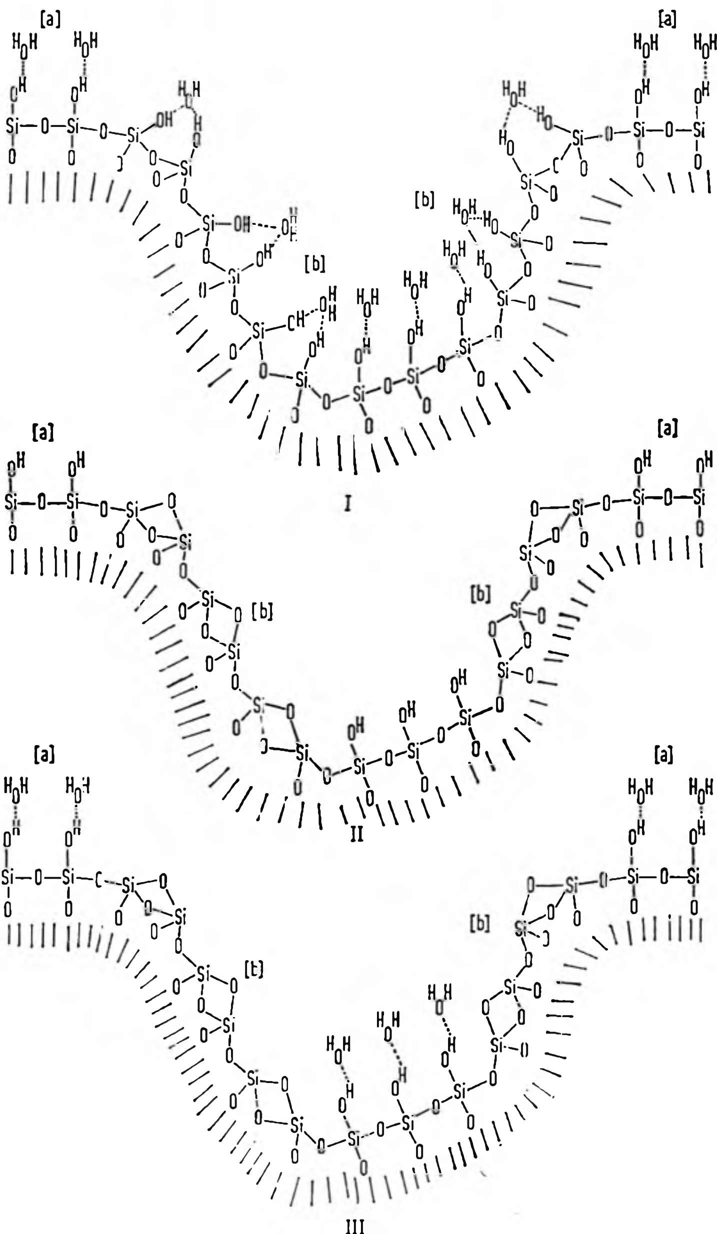


Fig. 8. Diagrammatic representation of a "footprint" and its deactivation. Position (a) represents the normal arrangement of silanol groups on the surface of silica gel. Position (b) represents the special arrangement of silanol and siloxan groups in the footprint.

I, active site. II, dehydrated surface. III, deactivated site.

if the trapped molecule in the adsorbent is an optical isomer, and if this isomer forms a racemic compound with its enantiomorph, then the attraction of the trapped L-isomer would be expected to be greater for its corresponding D- rather than for its L-adsorbate; that the reverse obtains implies footprints rather than attraction from the trapped molecules in the selective adsorbent.

2. If the trapped molecules led to selective attraction forces, it would be expected that an increase in the concentration of trapped molecules would give increased selectivity relative to the control. That there is no such correlation between the concentration of unextractable quinine and the adsorptive power of various quinine-selective adsorbents is seen from Table I.

3. Dry organic solvents passed through the selective adsorbents to remove water, abolished the selectivity relative to the similarly treated control, although the trapped quinine molecules in the selective adsorbents were not extracted by this treatment (Fig. 1). That changes in the surface area per g. of adsorbent were not an explanation of the loss of selectivity is indicated by the fact that both selective and control adsorbents before and after treatment adsorbed 5-aminoacridine, a molecule completely dissimilar to quinine, to the same extent (see Fig. 1).

4. Storage for some months of the selective adsorbents produced changes so that some of the previously "unextractable" quinine could then be extracted with methanol. This reduction in the concentration of trapped quinine *increases* rather than decreases the adsorptive power of the adsorbent for quinine while the adsorptive power of the similarly treated control did not change (Table II).

5. The change in selectivity when steam is forced through the adsorbent (Fig. 2) and the differences in the results of "dry" and "wet" heating of the adsorbent (Fig. 3) may be better explained by changes in surface footprints consequent on alteration of silanol groups (see later) rather than any explanation involving attraction forces from trapped molecules.

The Nature of the Footprints (Active Sites) and Modified Footprints

Implicit in the conclusion that footprints on the adsorbent surface and pores are responsible for the selectivity, is the acceptance that the arrangement of the silanol and hydrated silanol groups at these active sites differs from that of these groups in the control adsorbent surface or the ordinary surfaces of the selective adsorbents.

A diagrammatic representation of a hypothetical footprint resulting from removal of the foreign organic molecule from the surface of the silica gel is presented in Fig. 8, I; the differences in the relative orientation of some of the hydrated silanol groups in the footprint and general surface is depicted.

To account for the results presented herein, it is postulated that removal of water by treating the selective adsorbent with organic solvents, under the conditions specified, removes not only the free water but, *at the*

active sites also removes structural water molecules by converting some of the silanol groups (Fig. 8, I,b) to siloxane groups (Fig. 8, II,b). This can take place, we suggest, because of their relative orientation and because these groups probably have a higher energy than the normal silanol groups (see Fig. 8, I,a). When these "dehydrated" adsorbents are treated with water again, as occurs in the adsorption experiments, the silanol *but not the siloxane groups* will be rehydrated (see Fig. 8, III). On the other hand, the control adsorbents do not possess these abnormally orientated high energy hydrated silanol groups (cf. footprint and general surface of Fig. 8, I of the selective adsorbent), and when dehydrated with organic solvents and then rehydrated, the silanol groups will be regenerated mainly as in the original gel. Thus the adsorptive power of the control will be unchanged by treatment with the organic solvents whereas that of the selective adsorbent will be decreased to a level similar to that of the control.

The above argument implies that upon drying, a selective adsorbent should show a greater loss of water than a control adsorbent; the results in Table III agree with this deduction.

It also follows that the silanol-bound water plays an important role in the preservation of the high energy silanol groups in the footprint. The difference in the rate at which dry heat and heating the adsorbent in water causes loss of selectivity (Fig. 3) may be explained as follows: Water is bound firmly to silanol groups and is therefore slowly removed and in consequence the "dehydration" of silanol to siloxane groups at the active site upon dry heating at 90° is also slow. Although bombardment of the adsorbed water at the active site with water molecules permits hydration of the silanol groups, it involves interchange of water molecules and removes the protection of the adsorbed water at the high energy silanol bonds with the resultant effect of converting these to siloxane bonds with attendant loss of selectivity. A similar explanation accounts for the rapid loss of selectivity upon passing steam through the adsorbent (see Fig. 2).

The gradual loss of selectivity on storage (Beckett and Anderson, 1959) may also be explained by a slow conversion of some silanol to siloxane groups. During storage, it is to be expected that, as in the initial formation of the gel, there will be some slight breaking and making of bonds. This will result in some of the trapped molecules becoming less firmly enclosed in the silica lattice and so re-extraction of stored selective adsorbent will remove some previously trapped molecules with the formation of new active sites (see Table III).

Active Sites in Selective Adsorbents as Model Drug Receptors

Since suitable biologically-active reference molecules may be used to make the footprints in the surface of the adsorbent, and physically bound water on the silica surface plays an important role in the selectivity, these footprints may be regarded as simple models of drug receptors and enzyme active sites. Because the adsorption of some cinchona alkaloids to certain selective adsorbents is reversible (Beckett and Anderson, 1957),

then water molecules bound to the high energy silanol bonds must be retained during the adsorption of these alkaloids to their respective footprints. Consequently, binding of these molecules to active sites in selective adsorbents involves substantial binding to oriented hydroxy groups of adsorbed water molecules rather than a simple binding to a surface containing silanol groups; approximation to a biological surface is therefore closer than is first apparent.

As with selective adsorbents, adsorbed water is intimately involved in the structure and stabilisation of biological surfaces. For instance, X-ray diffraction and infra-red measurements on collagen (Harrington and Von Hippel, 1961) show that crystalline portions of the structure are probably stabilised by doubly hydrogen-bonded water bridges giving rise to continuous chains of structurally incorporated water along the fibre axis in the diffracting regions (see I).

```
          C              C              C
          ||             ||             ||
H–O–H...O...H–O–H...O...H–O–H...O
```

(I)

Fraser and Macrae (1959) concluded that the bound water molecules are primary singly bonded to the carbonyl groups which project radially outward from the collagen molecules.

Further similarities between the characteristics of active sites of selective adsorbents and those of biologically active sites are as follows:

1. Enzymes and apparently many drug receptors differentiate between enantiomorphic forms of compounds (Beckett, 1959) as do footprints in selective adsorbents when asymmetric molecules are used to make footprints (Beckett and Anderson, 1959).

2. Changes in certain groups in enantiomorphic pairs of compounds may alter the affinity to biological receptors but the relative affinity in the stereochemical sense is unaltered; the same applies with stereoselective adsorbents (see Table IV) (Anderson, 1961).

3. Enzyme active sites may be deactivated by heating, treatment with organic solvents and contact with strong acids (Dixon and Webb, 1960) and these effects are irreversible because of alteration of the surface characteristics; similarly, active sites in selective adsorbents may be altered by these treatments by altering the organisation at the surface and thus the hydration and binding forces (see Fig. 2, 3 and 4).

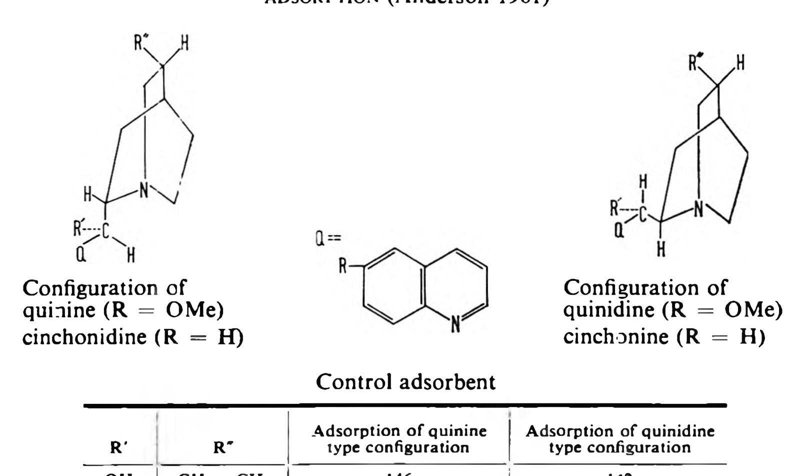
4. The interaction of enzymes and their substrates may be reversibly altered by changing the pH of the medium in which they interact (Dixon and Webb, 1960); similarly, the changes in interaction of quinine with a "quinine-footprint" is different from that of quinine with the control adsorbent upon changing the pH of the medium as indicated in Fig. 5 and these effects are reversible.

5. The degree of binding and the interaction of organic molecules with biological surfaces may be altered by the presence of salts. For

example the interaction of hexylresorcinol with the cytoplasmic membrane of *Escherichia coli* is increased by introducing sodium chloride into the biophase (Beckett, Patki and Robinson, 1959). Because the increase in the amount of quinine bound to a quinine-selective adsorbent is greater than that observed with the control up to concentrations of sodium chloride of 1 per cent, the interaction of quinine with active sites must have been increased (see Fig. 6). Further progressive increases in sodium chloride concentrations would be expected to increase the binding to both selective and control adsorbent by reducing the solubility of quinine in the aqueous medium; thus the contribution of the active site selective binding to the total binding will be reduced, so differences in selective and control will diminish as shown in Fig. 6.

TABLE IV

EFFECT OF CHANGES OF CERTAIN GROUPS IN THE ADSORBATE MOLECULES ON THE ADSORPTION (Anderson 1961)



Control adsorbent

R′	R″	Adsorption of quinine type configuration	Adsorption of quinidine type configuration
OH	$CH = CH_2$	146	142
H	$CH = CH_2$	212	210
H	COOH	134	134

Quinine-selective adsorbent

R′	R″	Adsorption of quinine type configuration	Adsorption of quinidine type configuration
OH	$CH = CH_2$	394	232
H	$CH = CH_2$	480	410
H	COOH	240	220

All adsorption figures are calculated at an equilibrium concentration of 20×10^{-5} M/kg. of adsorbent.

We conclude, therefore, that active sites in stereoselective adsorbents may be used as rough models of active sites in enzymes and of drug receptors in addition to the proven use of these adsorbents in the determination of configuration of organic molecules. The probability that the stereoselective adsorbents may also act as catalysts (cf. oriented bonding of molecules to massive catalysts in hydrogenation) because of

the oriented binding in the active sites, is also receiving attention as is their use in separation techniques.

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The paper was presented by MR. YOUSSEF.

THE STRUCTURES OF THE ALKALOIDS FROM *MITRAGYNA* SPECIES OF GHANA

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The known alkaloids, rhynchophylline, isorhynchophylline, mitraphylline and rotundifoline, were isolated in investigations on *Mitragyna* species of Ghana (Beckett, Shellard and Tackie, 1963). While the work was in progress, the structure of rhynchophylline and isorhynchophylline was elucidated as (I) (Seaton, Nair, Edwards and Marion, 1960); these alkaloids were shown to be isomeric about positions C(3) or C(4). More recently Finch and Taylor (1962) have provided convincing evidence that they are isomeric about C(3) and that the groups at C(15) and C(16) are *trans*. Meanwhile Wenkert, Wickberg and Leicht (1961) from N.M.R. spectra have deduced a *cis* arrangement of methoxymethylene and ester group about the double bond.

[I]

[II]

Recently the structure of mitraphylline has been established as (II) (Seaton, Tondeur and Marion 1958). Because oxidative rearrangement of the indole ajmalicine with known *trans* D/E junction gave mitraphylline (and isomitraphylline), this alkaloid must have the *trans* D/E junction (Shavel and Zinnis 1962, Finch and Taylor 1962). Evidence for the *trans* C/D junction was also presented.

Rotundifoline, isolated from *M. rotundifoline* has previously been allocated the molecular formula $C_{22}H_{26}O_5N_2$ (Barger, Dyer and Sargeant, 1939; Ongley 1950). Hendrickson (1962) assumed this was correct and from available evidence tentatively suggested formula (III).

On more recent evidence, the molecular formula has been amended to $C_{22}H_{28}O_5N_2$ by Beckett and others (1963). Acid hydrolysis of rotundifoline yielded an alkaloid devoid of a methoxy group and further rendered formula (III) untenable. On the basis of ultra-violet and infra-red spectroscopy studies, N.M.R. spectra and mass spectrometry, we have now established the structure of rotundifoline as (IV), hydrolysis yielding the aldehyde (V) (Beckett and Tackie, 1963).

From *M. stipulosa* and *M. ciliata* another oxindole alkaloid $C_{22}H_{28}O_5N_2$, was isolated by Beckett, Shellard and Tackie (1963). Unlike rotundifoline, of the same molecular formula, it was soluble in sodium hydroxide solution and its ultra-violet spectrum changed upon changing the pH of the solutions. Recently this alkaloid, now designated

[III] [IV]

isorotundifoline, was shown to be an isomer of rotundifoline. On the basis of pK_a values, rates of quaternisation, the formation of the same quaternary ammonium iodide from either isomer, ultra-violet and infra-red spectroscopy under different conditions, we conclude that these alkaloids, unlike rhynchophylline and isorhynchophylline, are isomeric about C_4.

Two new oxindole alkaloids, $C_{23}H_{30}O_5N_2$, designed rhynchociline and ciliaphylline, were isolated from *M. ciliata* by Beckett, Shellard and Tackie (1963). It was subsequently found that isomerisation of ciliaphylline with acetic acid gave rhynchociline. Chemical and physico-organic data indicate that these isomers may be represented as (VI).

[V] [VI]

[VII]

Available evidence indicates that the new indole alkaloid, mitraciliatine, probably has a structure closely related to mitragynine whose structure (VII) has been recently suggested by Joshi, Raymond-Hamet and Taylor (1963).

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The paper was presented by DR. BECKETT.

A RAPID METHOD FOR THE DETERMINATION OF GRISEOFULVIN IN FERMENTER BROTH

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Received May 1, 1963

A method has been devised for the determination of griseofulvin in fermenter broths. This is based on the conversion of griseofulvin to isogriseofulvin with methane sulphonic acid in methanol. The reaction is followed by measuring the resulting change in ultra-violet absorption. The method is rapid, accurate and suited to the routine examination of a wide variety of samples.

Griseofulvin in methanol shows two absorption maxima at 236 mμ (ϵ = 22,900) and 291 mμ (ϵ = 24,000). For the estimation of griseofulvin the method of Ashton and Tootill (1956) utilises the peak at 291 mμ and applies a seven-point statistically derived correction to allow for irrelevant absorption in the fermenter broth extract. The procedure requires lengthy evaluation of the experimental results and is ill suited to the daily examination of large numbers of samples. More serious, however, is the failure to differentiate between griseofulvin and such closely related compounds as dihydro-, dehydro-, tetrahydro- and dechlorogriseofulvin, griseofulvic acid and isogriseofulvin, all of which are returned as griseofulvin and all of which have been isolated from fermenter broth (unpublished observations). The present work was undertaken with the object of developing a rapid and simple assay specific for griseofulvin and applicable to a wide range of fermenter broth samples, including those typifying complex media and fermentation conditions.

Experimental

The change from griseofulvin to isogriseofulvin may be shown thus:

and a process for the isomerisation using hydrochloric acid in methanol has been described by Grove, MacMillan, Mullholland and Thorold-Rogers (1952). Griseofulvin and its isomer show significant differences in absorption between 260 and 280 mμ (Fig. 1) and an assay based on this conversion is obviously possible if a reasonable and consistent proportion of the griseofulvin is capable of being converted to its isomer.

Untreated fermenter broth is unsuitable for spectrophotometric measurement and extraction of griseofulvin therefrom is necessary. Of

various solvents tried, ethyl acetate was selected, as it had satisfactory optical transmission between 260–280 mμ and griseofulvin was reasonably soluble in it. For the isomerisation of griseofulvin to isogriseofulvin ethanolic solutions of hydrochloric acid, sulphuric acid and methane sulphonic acid all gave complete conversion of 1 mg. of griseofulvin in ethyl acetate in under 1 hr. at 20° and on account of the speed of reaction and ease of preparation, 2N methane sulphonic acid was chosen for routine use.

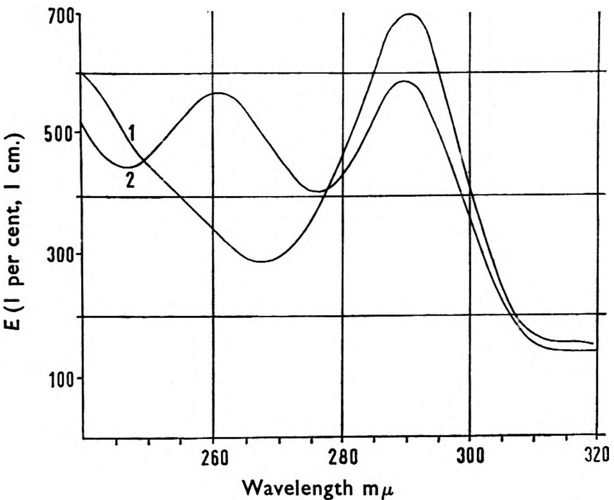

FIG. 1. Ultra-violet absorption curves for griseofulvin (1) and isogriseofulvin (2) in methanol.

Influence of water on the rate of isomerisation. It was found that water inhibited the rate of isomerisation to a marked degree, but the addition of anhydrous sodium sulphate to the sample before extraction overcame the difficulty.

TABLE I

INFLUENCE OF STRUCTURALLY RELATED IMPURITIES

Impurity	Assay by proposed method calculated as griseofulvin, per cent w/w	Assay by ultra-violet (B.P. 1963) method calculated as griseofulvin, per cent w/w
Isogriseofulvin	Nil	82
Tetrahydrogriseofulvin ..	Nil	80
Dihydrogriseofulvin	−28·5	89
Dechlorogriseofulvin ..	110	100
Griseofulvic acid	−25	100
Dehydrogriseofulvin	−80	100

Influence of structurally similar impurities. Samples of impurities known to be present in fermenter broth were assayed by the proposed method. The results (Table I) show the method to be unaffected by either tetrahydro- and isogriseofulvin; the remaining impurities contribute some measure of interference, but with the exception of dechlorogriseofulvin,

the error introduced is far less than with the direct spectrophotometric procedure.

METHOD

Reagents

Standard griseofulvin solution. Dissolve pure griseofulvin (100 mg.) in ethyl acetate (100 ml.)

2N *Methane sulphonic acid.* Dissolve methane sulphonic acid (19·2 g.) in methanol A.R. (100 ml.)

Preparation of calibration graph. Transfer aliquots of 0·5, 1, 2, 3 and 4 ml. of standard griseofulvin solution to each of two groups of five 100 ml. volumetric flasks and add ethyl acetate to bring the volume in each flask to 5·0 ml. The first group of flasks represents the standard series; the second group the blank series. To each flask of the sample series, add 2N methane sulphonic acid (5·0 ml.). Mix and allow to stand at room temperature (20° $\pm$ 3°) for 30 min. Dilute the contents of both series to 100 ml. with methanol A.R. and measure the extinction of each sample solution against the appropriate blank at 266 mμ, construct the calibration graph.

Sample assay. Transfer well mixed whole broth (2·5 g.) to a 150 ml. flask and add methanol A.R. (5·0 ml.) and anhydrous sodium sulphate (2 g.), stopper and shake vigorously to obtain an even dispersion. Add ethyl acetate (45·0 ml.), shake vigorously for 2 min. and filter. Transfer 5·0 ml. of the filtrate to each of two 100 ml. volumetric flasks and proceed as described in the preceding section.

RESULTS AND DISCUSSION

Samples chosen to cover a wide variation of media composition and fermenter conditions were analysed by the proposed method, both before and after fortification with known quantities of pure griseofulvin. The results shown in Table II illustrate quantitative recovery of the added griseofulvin. The proposed method gives results well within the range of accuracy usually considered acceptable for fermenter broth samples, and although not completely specific for griseofulvin, has considerable

TABLE II

RECOVERY EXPERIMENTS ON FERMENTER BROTH FORTIFIED WITH ADDED GRISEOFULVIN

Sample No.	Griseofulvin initially present mg.	Added mg.	Total mg.	Found mg.	Recovery per cent w/w
1	8·13	Nil	8·13	8 13	100
2	8·27	2·58	10·85	10·85	100
3	8·05	8·06	16·11	16·17	101
4	7·54	18·75	26·29	25·08	95·5
5	8·32	Nil	8·32	8·32	100
6	7·43	2·56	9·99	9·56	95·5
7	7·21	7·43	14·64	14·22	97
8	7·58	17·81	25·39	24·65	97
9	Nil	5·66	5·66	5 50	97
10	Nil	12·4	12·4	11 8	95
11	Nil	2·33	2·32	2 32	100
12	Nil	7·33	7·33	7 03	96
13	Nil	17·46	17·46	16 76	96

advantage over previously published procedures both in day to day control of plant manufacture and in assessing the results of mutant screening experiments.

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A QUANTITATIVE CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PURITY OF GRISEOFULVIN

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Received May 1, 1963

A chromatographic assay for the determination of griseofulvin is described, employing a hexane: methanol: chloroform: water partition system supported on Celite. Chromatographic separation is followed by ultra-violet measurement of the eluate fractions at 291 mμ. The system enables the determination of griseofulvin to be made in the presence of tetrahydrogriseofulvin, dehydrogriseofulvin, dihydrogriseofulvin, isogriseofulvin, dechlorogriseofulvin and griseofulvic acid.

METHODS for the determination of griseofulvin in fermenter broth fail to differentiate completely between griseofulvin and structurally similar contaminants which are also present (unpublished observations). The assay of broth samples is normally undertaken with the object of following the progress of the fermentation where this lack of specificity is not a serious handicap.

The presence of small amounts of these closely related substances in the final product would, however, go undetected by such methods and it was therefore considered desirable that a specific assay for griseofulvin be developed.

EXPERIMENTAL

The physical properties of griseofulvin and of some of the likely contaminants are listed in Table I. In view of their close similarity,

TABLE I

PHYSICAL PROPERTIES OF GRISEOFULVIN AND SOME LIKELY CONTAMINANTS

Compound	Melting-point °C	Ultra-violet absorption λmax mμ	Log ε	Infra-red absorption C=O region υmax. cm.$^{-1}$	Specific rotation $[\alpha]_D^{20}$
Griseofulvin	216	324 291 236	3·79 4·38 4·36	1,653 1,701	+356°
Dihydrogriseofulvin	198	323 288 234	3·70 4·31 4·10	1,680 1,715	−20°
Tetrahydrogriseofulvin	181	322 287 234	3·68 4·28 4·10	1,700	−34°
Dechlorogriseofulvin	179	288 250	4·40 4·18	1,652 1,695	+400°
Isogriseofulvin	199	325 291 262 234	3·70 4·31 4·39 4·31	1,654 1,696	+215°
Griseofulvic Acid	255–258	326 291 267 236	3·72 4·40 4·30 4·17	1,654 1,670 1,695	+399°

preliminary separation of the griseofulvin is obviously essential in any analysis specific to this substance. Previous experience with partition chromatography on Celite suggested a possible solution to the separation of griseofulvin from other likely impurities.

The solvent system used was obtained by shaking together methanol: water: hexane: chloroform (8 : 2 : 9 : 1). The lower layer of the mixture was employed as stationary phase supported on the Celite column packing. The upper layer was used to develop the column. The progress of the chromatogram was followed by measuring the extinction of successive 10 ml. fractions of column eluate at 291 mμ, the curve relating extinction to volume of eluate for pure griseofulvin is shown in Fig. 1.

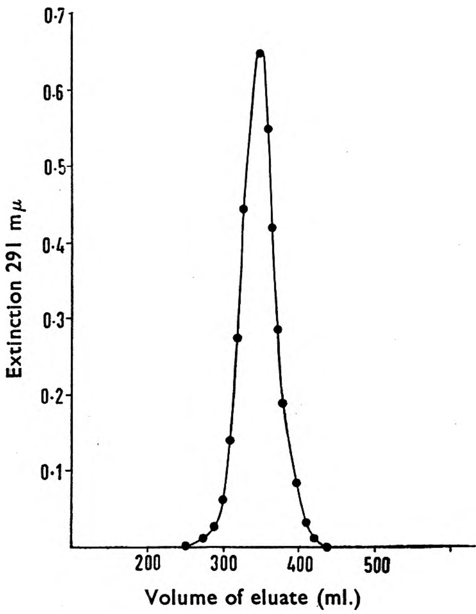

FIG. 1. Curve relating extinction with volume of eluate for pure griseofulvin.

Reagents

Prepared Celite. Stir Celite 545 (500 g.) intermittently for 12 hr. with concentrated hydrochloric acid (2 litres). Decant the hydrochloric acid and suspend the residue in 1 litre of water. Filter through a Buchner funnel, wash the residue with water until free from acid, wash with methanol (500 ml.) then methanol/ethyl acetate (1 : 1) (1 litre). Dry the residue in an oven at 100°. Store in well stoppered jars.

Procedure

Preparation of solvent system. Shake together methanol A.R. (800 ml.), water (200 ml.), n-hexane (900 ml.) and chloroform A.R. (100 ml.) and allow to separate. The upper layer is the eluent phase; the lower, the stationary phase.

Preparation of sample and standard. Dissolve about 20 mg. accurately weighed in the stationary phase and dilute to 25 ml. in a volumetric flask with the same mixture. Prepare a standard solution of pure griseofulvin in a similar manner.

Preparation of chromatographic column. Mix prepared Celite (15 g.) with stationary phase (7·5 ml.) and pack into a chromatographic column (70 cm. in length 2·2 cm. internal diam. fitted with a sinter plate) in portions of about 3 g., packing down firmly with a tamper between each addition. To a further 2 g. of prepared Celite add sample solution

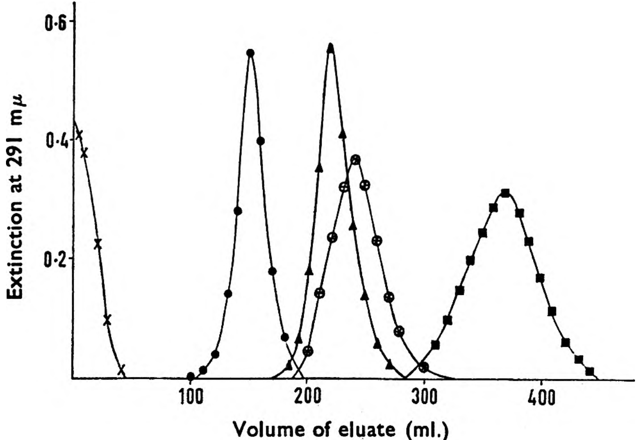

FIG. 2. Curves relating extinction volume for griseofulvin and some commonly encountered impurities. ×——× Tetrahydrogriseofulvin. ●——● Dihydrogriseofulvin. ▲——▲ Isogriseofulvin. ○——○ Dechlorogriseofulvin. ■——■ Griseofulvin.

(1·0 ml.), mix thoroughly and transfer the mix quantitatively to the top of the stationary phase in the column. Carefully add eluent phase until the stationary phase is covered to a depth of about 50 cm. and adjust the flow of eluate from the column to about 10 ml./75 sec. Collect 50 successive 10 ml. fractions of eluate in 6 in. × 1 in. stoppered test tubes. Measure the extinction at 291 mμ of each fraction against eluent phase in the reference cell. Use 1 cm. cells. Repeat the chromatogram using 1·0 ml. of standard griseofulvin solution.

Then, per cent griseofulvin in the sample $= \dfrac{\epsilon_a \cdot w_s \cdot 100}{\epsilon_s \cdot w_a}$ where ϵ_a and ϵ_s are the sums of extinction values under the sample and standard peaks respectively: w_a = weight of sample (mg.). w_s = weight of standard griseofulvin (mg.)

Samples of tetrahydrogriseofulvin, dihydrogriseofulvin, dehydrogriseofulvin, isogriseofulvin, dechlorogriseofulvin and griseofulvic acid were assayed as described above and the relationships between extinction at 291 mμ and volume of eluate for the less polar group of compounds are illustrated in Fig. 2. It is clear that none of the above compounds

interferes with the assay of griseofulvin by the method described and this was confirmed by the assay of artificial mixtures of pure griseofulvin with each of the above mentioned impurities. Quantitative recovery of the griseofulvin was obtained in every instance.

TABLE II

COMPARISON OF RESULTS OBTAINED USING THE PROPOSED METHOD WITH THOSE OF THE DIRECT ULTRA-VIOLET ASSAY

Sample	Griseofulvin content per cent w/w		Major impurities detected
	Proposed method	U.V. method (B.P. 1963)	
Pharmaceutical Grade Griseofulvin			
1	97·0	98·2	
2	99·5	99·0	
3	97·0	97·6	
4	100·0	100·2	
5	99·6	100·0	
6	98·4	99·0	
7	99·5	98·6	
Crude Griseofulvin			
8	96·0	96·6	Dechlorogriseofulvin
9	90·5	97·8	"
10	95·0	97·2	"
11	96·0	97·2	"
12	96·0	97·5	Dechlorogriseofulvin + Dehydrogriseofulvin
Griseofulvin Residues			
13	51·7	95·0	Dechlorogriseofulvin + Dihydrogriseofulvin
14	81·0	98·0	Dechlorogriseofulvin
15	83·5	91·5	"
16	44·0	87·2	Griseofulvic Acid
17	55·0	100·0	Dehydrogriseofulvin

Results and Discussion

The proposed method has been applied to a series of samples of both pharmaceutical and crude grades of griseofulvin, and to material isolated from mother liquors at various stages of the purification process. The results compared with those using the direct ultra-violet absorption procedure proposed for the B.P. 1963 are shown in Table II and illustrate the value of the method for both control of pharmaceutical grade griseofulvin and in process development studies. Much information is gained about the nature and quantity of any impurity present and although the method as described is not recommended for the accurate determination of trace impurities in the pharmaceutical grade, simple adaptations of the technique that make this possible will be the subject of a further publication.

The papers were presented by MR. HOLBROOK.

THE *MITRAGYNA* SPECIES OF GHANA

THE ANATOMY OF THE LEAVES OF *Mitragyna stipulosa* (D.C.) O. KUNTZE AND *Mitragyna ciliata* AUBR. ET PELLEGR.

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The morphology and detailed anatomy of the leaves, stipules and young stems of *Mitragyna stipulosa* (D.C.) O. Kuntze, and *Mitragyna ciliata* Aubr. et Pellegr. have been described. Although the histological feaures are almost identical, the leaf of *Mitragyna stipulosa* has a typical shade leaf structure and that of *Mitragyna ciliata* a typical sun leaf structure.

Mitragyna stipulosa and *Mitragyna ciliata* are trees which may grow to a height of about 35 metres. Examination of the leaves of both species for alkaloidal content showed distinct differences between them (Beckett, Shellard and Tackie, 1963a,b). Morphologically it is extremely difficult to distinguish them. The two species were, until 1936, regarded as a single species, *Mitragyna macrophylla*, known throughout West Africa as bahia, when Aubreville drew attention to slight differences between the flowers of those trees growing in the closed rain forests and those growing in the Savanah. In the former, which is named *M. stipulosa*, the gamosepalous calyx has an entire margin which is glabrous. It is larger than the floral bracteole, thus being readily visible. In the latter, which he named *M. ciliata*, the calyx has an undulating or even slightly lobed margin which is ciliate. It is much shorter than the bracteole and is is thus not easily seen in the flower. A further difference he considered to be of significance, is a phytogeographical one. Both species are gregarious and are found growing in distinct and well defined localities. According to Irvine (1961) *M. stipulosa* grows in low lying swampy parts of the fringing and savannah forests while *M. ciliata* grows in freshwater swamps in closed (rain) forests.

However, the habitats from which the materials we have examined were collected were opposite to those given by Irvine. Attempts to locate species in the regions described by Irvine were not successful.

Since the anatomical structure of the leaves of these two species had not been described it was thought desirable to make a comparative study of them.

MATERIALS

The leafy twigs of both species were obtained from January 1961 to June 1962. *M. stipulosa* specimens were from trees growing in the forest near to the campus of the Kwame Nkrumah University of Science and Technology, Kumasi in the Ashanti region, well within the closed (rain) forest zone.

M. ciliata specimens were from trees growing in the vicinity of Tarkwa in the Western Region. This is in the fringing and savannah forest zone.

The identity of both species was confirmed by Mr A. Enti, Government Silviculturist, and the twigs, leaves and flowers by comparison with the specimens in the Herbarium in the Royal Botanic Gardens, Kew.

In the following description, where the features of the two species are indistinguishable, a single account is given.

MACROSCOPICAL FEATURES

The young stems bear 6–8 leaves arranged in opposite pairs, with interpetiolar stipules. The leaves are simple and are up to 50 cm. long and 32 cm. wide. When fresh the younger leaves are reddish, the older a bright green. Dried, in the shade, their colour changes to a dull or brownish green. The lamina of the younger leaves is round or broadly oval and of the larger leaves, oval to broadly elliptical. The margin is almost entire or undulate, the apex is rounded or very bluntly acuminate and the base is rounded though frequently slightly decurrent.

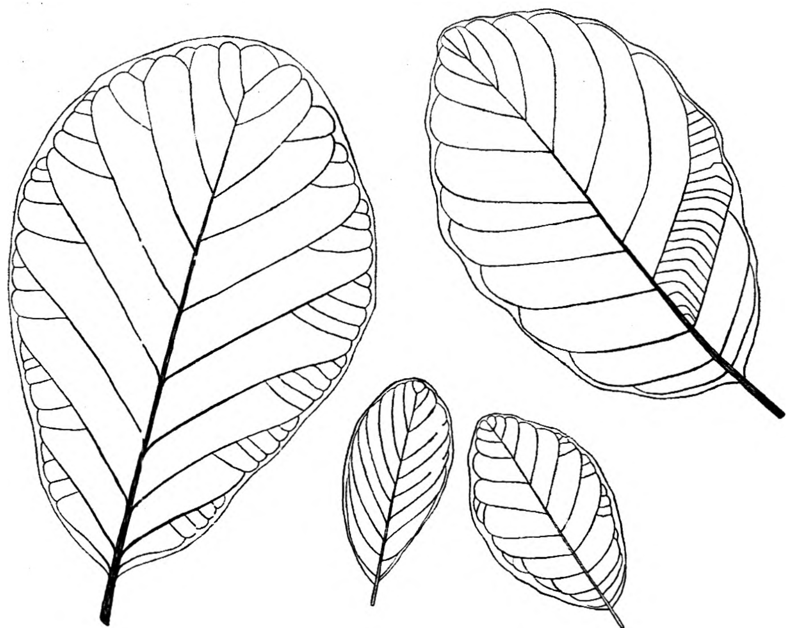

FIG. 1. Leaves of *Mitragyna stipulosa* (D.C.) O. Kuntze and/or *Mitragyna ciliata* Aubr. et Pellegr × $\frac{1}{6}$.

The upper surface shows distinct pinnate venation but this is more marked on the lower surface. At the base of the lamina, the upper surface of the midrib is grooved and may be up to 6 mm. wide. The midrib and the 8–10 lateral veins on each side are reddish brown. In some leaves the lateral veins leave the midrib at a wide angle, especially near the

base; in other leaves the angle is narrow. They are straight or only slightly curved until they get near to the margin when they curve upwards towards the margin and subsequently anastomose to form a marginal vein (Fig. 1A,B). Secondary veins run between the lateral veins and are roughly parallel to each other.

The upper surface of the leaves is glabrous. On the under surface the midrib and lateral veins are pubescent but no trichomes are present in the interneural parts. Some of the trichomes are long and yellow and on the small young leaves give a densely pubescent appearance.

The dark reddish brown petioles which measure from 0·5 cm. in length in the young leaves to 4·0 cm. in the larger leaves may be flattened and twisted and up to 6 mm. wide at the base. The glabrous upper surface is grooved longitudinally, while the lower surface is faintly ridged longitudinally and is slightly, or at the base distinctly, pubescent. The petioles of the young leaves have a densely pubescent lower surface.

The stipules may be up to 7·5 cm. long and 5·5 cm. wide. Before the emergence of the new shoot the stipules are adpressed to each other. One of the two stipules is slightly larger and its margin folds over the other stipule to seal in the young leafy shoot. The young stipules are round and dark red, the larger ones green and obovate or suborbiculate, both kinds tapering towards the base; margin entire; upper (inner) surface glabrous; lower (outer surface) distinctly pubescent, the yellow trichomes being prominent in the lower half, which in the young stipules is densely pubescent. At the base of the stipule on its inner surface, there are two or three rows of brown elongated protuberances which produce a sticky transparent secretion as a protectant to the young shoot. In contact with water the secretion becomes a white latex. According to Aubreville (1936) the stipules of *M. stipulosa* are more pubescent than those of *M. ciliata*: we did not detect this. The venation, although palmate appears to be parallel, there being up to 26 veins which are conspicuous but not prominent. They curve outwards towards the margin where they anastomose (Fig. 6A).

The young stems or twigs are dark brown and somewhat flattened; they may be up to 0·75 cm. wide and longitudinally grooved, with the entire surface covered with numerous trichomes.

The leaves stipules and twigs are odourless but have a slightly bitter taste.

Microscopical Features

The Leaf

Lamina. The upper epidermis consists of a single layer of polygonal cells covered with a thick ridged cuticle which gives a characteristic striated appearance to the epidermis. The striations of *M. stipulosa* are characterised by being short, irregular in outline and frequently confined to individual cells. Those of *M. ciliata* are long and undulating and are continuous over several contiguous cells (Fig. 2, A–D). The outer walls of the cells are thickened but the anticlinal walls, which are straight or only slightly sinuous, are not thickened. No stomata or trichomes are

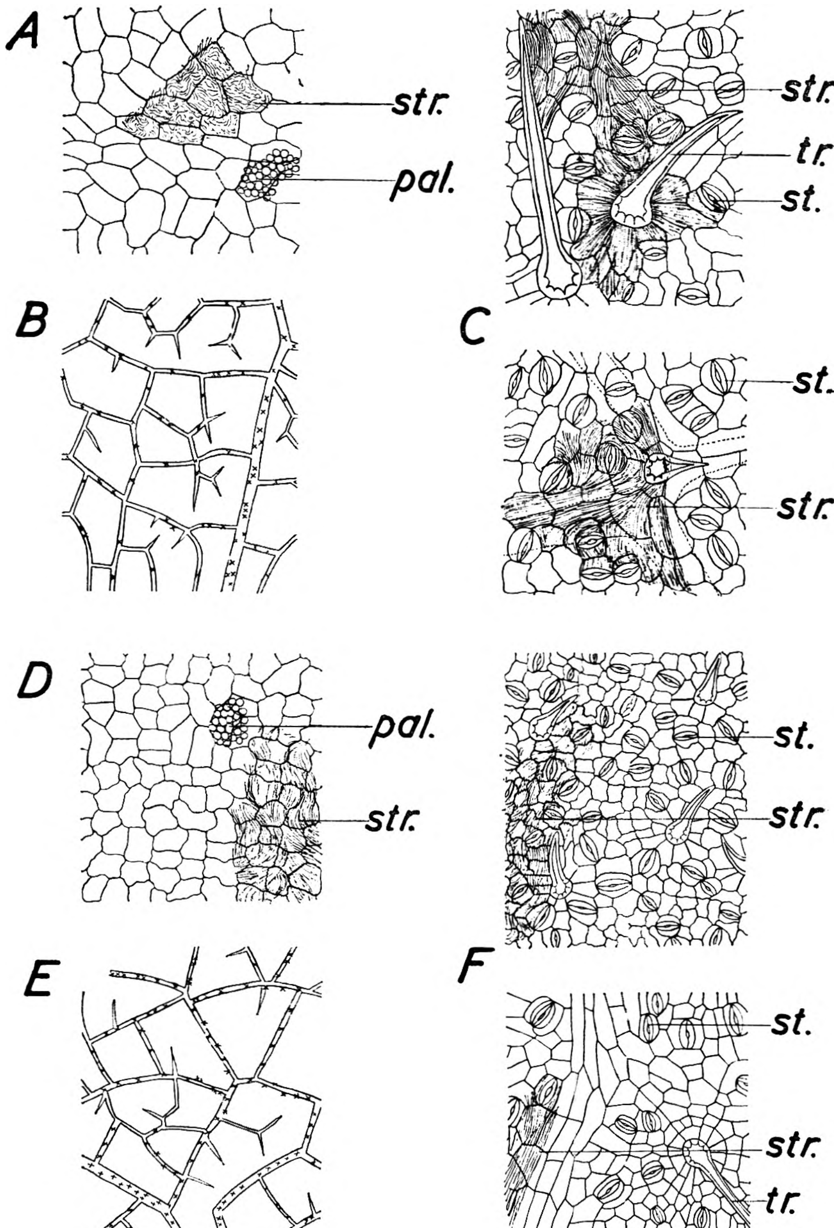

FIG. 2. *M. stipulosa*, leaf, A, upper epidermis, surface view × 160; B, veinlet distribution × 40; C, lower epidermis, surface view × 160; D, *M. ciliata*, leaf, upper epidermis, surface view × 160; E, veinlet distribution × 40; F, lower epidermis, surface view × 160. pal., palisade; st., stomata; str., striations, tr., trichome. X represents crystals of calcium oxalate.

present. The epidermal cells are 20–55 μ long, 12–30 μ wide and 20–30 μ high for *M. stipulosa* and 15–50 μ long, 10–30 μ wide and 20–30 μ high for *M. ciliata.* Over the larger veins the cells are elongated parallel to the veins, measuring up to 75 μ long in both species.

The transverse sections of the two leaves are readily distinguishable.

M. stipulosa. The palisade occupies about one half of the width of the mesophyll and consists of two or occasionally three rows of thin-walled cylindrical cells, those of the outer row being longer and narrower than the others. Frequently the cells of the inner row appear funnel shaped. The palisade is continuous over the smaller but not the lateral veins. The palisade ratio is 11·5–**13·5**–16·2.

The spongy mesophyll consists of thin-walled rounded to elongated, or occasionally funnel-shaped cells which are unlikely to function as "collecting cells" ("Sammenzellen," Haberlandt, 1909), since there is no clear relationship between them and the adjacent tissues. Some cells have thick highly refractive walls (Fig. 3, G).

M. ciliata. The palisade, which occupies about two-third to three-quarters of the width of the mesophyll, consists of four and occasionally three or five rows of thin-walled cylindrical cells, most of which are of equal length except where there are three rows of cells, when, in the outer row, cells are about twice as long as the others. Very few funnel-shaped cells occur but many of the cells of the inner row are dumb-bell-shaped. It is not continuous over the lateral or the smaller veins. The palisade ratio is 8·75–**10·4**–16·3.

The spongy mesophyll consists of thin-walled rounded to elongated cells, some of which are dumb-bell-shaped. Some cells have thickened highly refractive walls. There are few air spaces in the region adjacent to the lower epidermis and these regularly arranged, frequently dumb-bell-shaped cells might be regarded as a palisade of one or two rows (Fig. 4, D).

The appearance of the transverse section of the leaf of *M. stipulosa* is typical of a leaf which grows in the shade while that of *M. ciliata* corresponds with that of a leaf which grows in direct sunlight. Such differences have been noted in other plants by Nordhausen (1903, 1912). If these differences correspond to variations in light intensity this is supported by the nature of the habitats in which these species grow.

The lower epidermis in both species consists of a single layer of polygonal cells, the outer walls of which are only slightly thickened. The epidermis is covered with a thin ridged cuticle. The striations are long and undulating and continuous over several contiguous cells. They are less conspicuous than on the upper epidermis. The unthickened anticlinal walls are more sinuous than those of the upper epidermis, except over the larger veins where the cells are straight-walled and elongated in the direction of the vein (Fig. 2, C and F).

M. stipulosa. The epidermal cells measure 20–60 μ long, 15–35 μ wide and 10–25 μ high, except for over the larger veins they may be up to 100 μ long. For *M. ciliata* measurements are 12–40 μ long, 8–30 μ wide and 10–25 μ high except over the larger veins where they may be up to

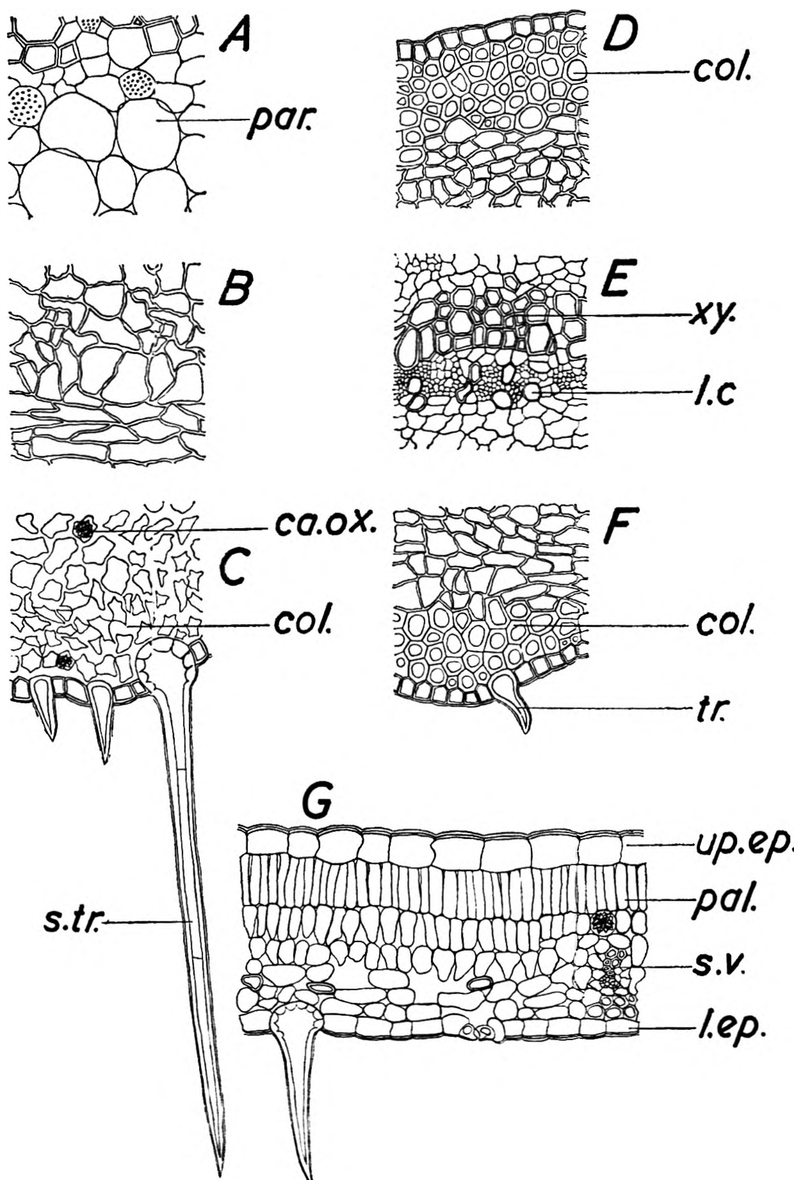

FIG. 3. *M. stipulosa*, t.s., midrib, A, pith near base × 160; B, cortical parenchyma × 160; C, lower epidermal region near base × 160; D, upper epidermal region near apex × 160; E, stele near apex × 160; F, lower epidermal region near apex × 160; G, t.s. lamina × 160. ca.ox., calcium oxalate; col., collenchyma; l.c., latex cell; l.ep., lower epidermis; pal., palisade; s.tr., septate trichome; s.v., small vein; tr., trichome; up.ep , upper epidermis; xy., xylem.

85 μ long. Except in the region of the veins there are numerous paracytic stomata. The stomatal indices are *M. stipulosa*, 14·4–**18·8**–20·2; *M. ciliata*, 16·2–**19·4**–22·2.

Numerous unicellular conical trichomes with thick slightly lignified walls and a slightly striated cuticle arise from cells near the veins. The basal portions of the trichomes are greatly enlarged, measuring 25–60 μ across, with thick strongly lignified walls having many conspicuous simple pits.

The trichomes of *M. stipulosa* measure 45–520 μ long and of *M. ciliata*, 45–600 μ long; in both species they are 7–25 μ wide near the base.

The larger trichomes have transverse septa and yellowish brown contents. The epidermal cells surrounding them are elongated and arranged in a stellate pattern. Trichomes also arise from the cells directly over the veins but there is no stellate arrangement of the adjacent cells (Fig. 2, C, F).

Cluster crystals of calcium oxalate accompany the veins (Fig. 2, B, F). The crystals occur in thin-walled parenchymatous cells which surround the veins. They are arranged in longitudinal files of up to 12 crystals and measure 4·5–17·5 μ in diameter for *M. stipulosa*, and 7·5–25 μ in diameter for *M. ciliata*.

The lateral and secondary veins consists of a few spirally and annularly thickened vessels and reticulately thickened tracheids together with a small amount of phloem. The lateral veins are surmounted on the upper and lower sides by parenchyma and collenchyma, the latter being adjacent to the epidermises. The vein islet numbers are *M. stipulosa*, 13·0–**16·4**–18·0; *M. ciliata*, 11·0–**13·3**–16·0.

Midrib. The upper epidermis consists of polygonal cells elongated parallel to the vein. The cell walls are slightly thickened with a fairly thick cuticle less ridged and less distinct than that covering the epidermis of the lamina. In the basal part the cells gradually change to resemble those of the upper epidermis of the petiole, the cuticle becoming smoother and the striations less obvious. Stomata and trichomes are absent.

In both species the lower epidermis is similar to the upper epidermis except that in *M. stipulosa*, the striations on the lower epidermis resemble those on the epidermal cells of the interneural lamina. Stomata are absent. Trichomes are numerous and resemble those on the secondary veins. There are two distinct size groups, those measuring 45–150 μ long and 7–12 μ wide near the base and those measuring 400–750 μ long and 20–30 μ wide near the base, and for the larger trichomes up to 75 μ across. (Figs. 3, C and 4, A).

The palisade of the lamina is discontinuous over the midrib. Extensive zones of collenchyma occur inside each epidermis. In the apical part of the midrib the collenchyma shows fairly even thickening (Fig. 3, D–F) but in the basal part where the cells are larger, there is extensive and irregular thickening on the tangential walls (Figs. 3, C and 4, A and B). Cluster crystals of calcium oxalate are present in some of the collenchyma and measure 4·5–17·5 μ diameter for *M. stipulosa* and 7·5–25 μ diameter for *M. ciliata*.

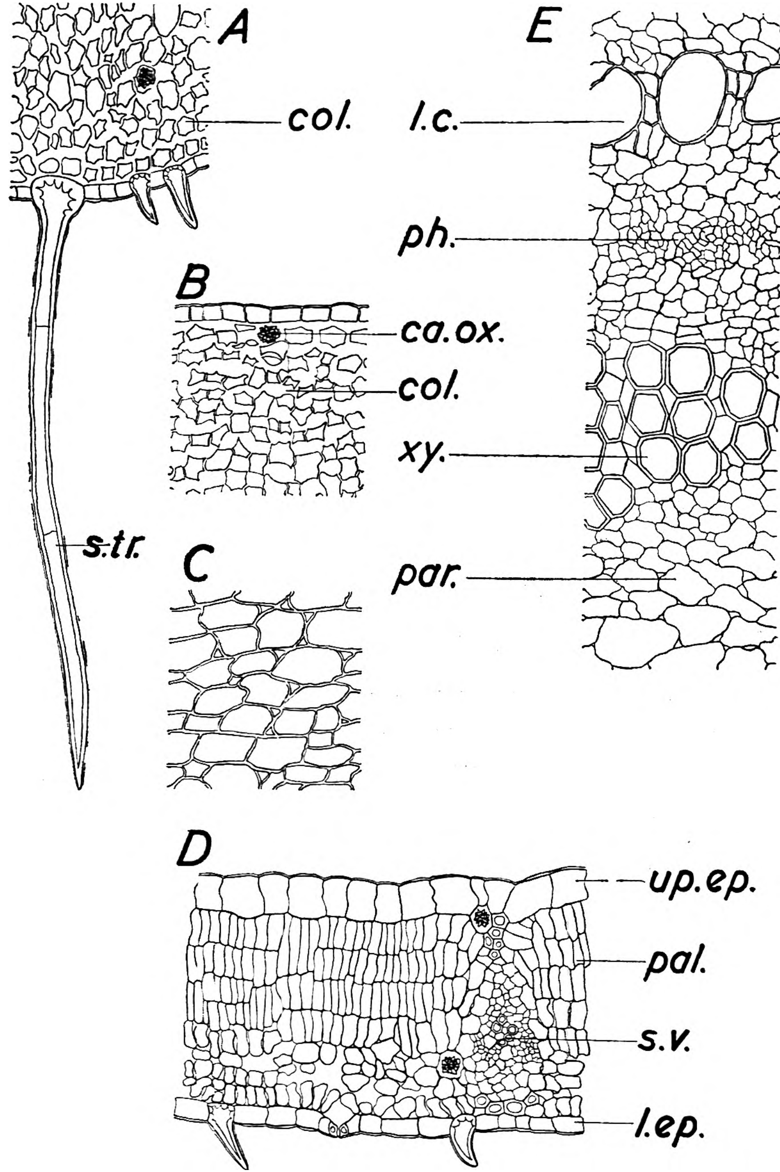

FIG. 4. *M. ciliata*, A, t.s., midrib, lower epidermal region near base × 160; B, t.s., petiole, upper epidermal region × 160; C, pith × 160; D, t.s., lamina × 160; E, t.s., midrib, stele × 160. ca.ox., calcium oxalate; col., collenchyma; l.c., latex cell; l.ep., lower epidermis; pal., palisade; par., parenchyma; ph., phloem (sieve tissue); s.tr., septate trichome; s.v., small vein; up.ep., upper epidermis; xy., xylem.

Thick-walled irregular shaped parenchyma lies beneath the collenchyma with no calcium oxalate (Fig. 3, B).

The endodermis is indistinct, being recognisable only by the occasional presence of small rounded starch grains.

The meristele is circular though the shape of the xylem and the presence or absence of additional meristeles, both outside the stelar region and in the pith, depends upon the position along the midrib. The additional meristeles may be either circular or arcuate and some of the arcuate meristeles on the pith are inverted. They frequently appear to be part of a discontinuous circular meristele (Fig. 5).

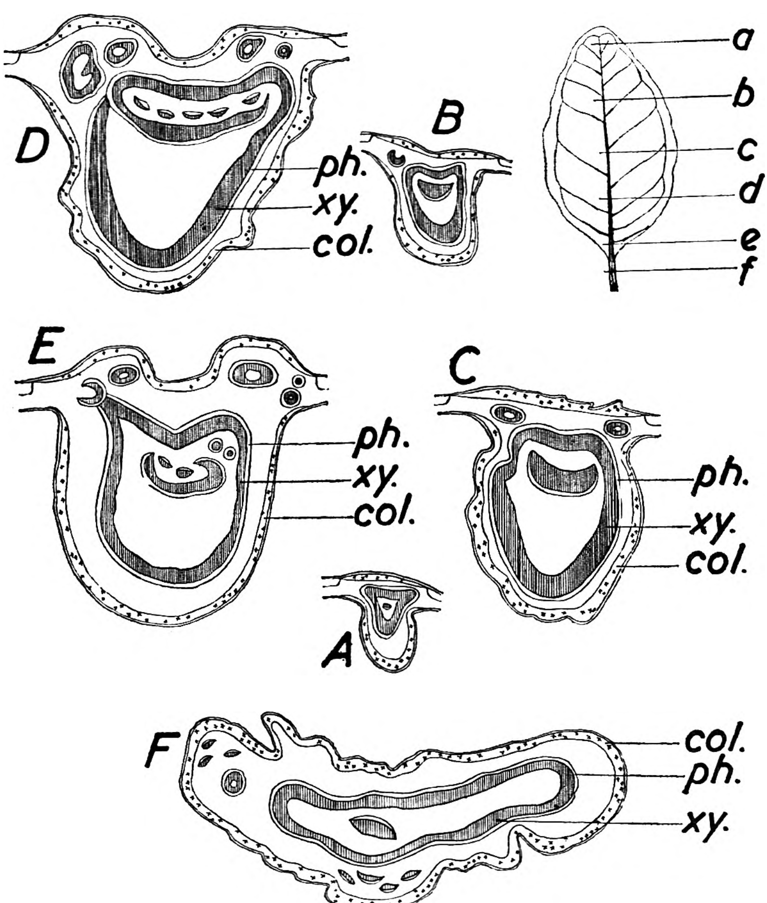

FIG. 5. *M. stipulosa*, A–E, t.s., midrib × 10; F, t.s.. petiole × 10. col., collenchyma; ph., phloem; xy., xylem.

The xylem consists of radial rows of lignified vessels which may be spirally, annularly, scalariformly or reticulately thickened or have elongated to oval, large bordered pits, reticulately thickened tracheidal vessels and xylem parenchyma. Xylem fibres are absent. Yellowish

brown contents occur in the xylem paranchyma, giving no colour with solution of ferric chloride.

The phloem consists of sieve tissue, phloem parenchyma and latex cells. Phloem fibres are absent. The latex cells have thickened walls and, in *M. stipulosa*, measure 20–35 μ in diameter and in *M. ciliata*, 40–65 μ in diameter. The yellowish brown latex is insoluble in light petroleum, ether and dilute acid but soluble in alcohol (96 per cent), chloroform and solution of sodium or potassium hydroxide. Starch and calcium oxalate are absent (Figs. 3, E and 4, E).

The pith consists of small thin-walled irregular shaped cells with few small intercellular spaces in all parts of the midrib except in the basal portion of that of *M. stipulosa* where the cells are large thin-walled and rounded with conspicuous simple pitting, the tissue containing many intercellular spaces (Figs. 3, A and 4, C). Starch and calcium oxalate are absent from the pith.

Petiole

The general anatomy of the petiole is similar to that of the midrib in the basal part of the leaf. The cells of both epidermises are elongated parallel to the long axis of the petiole and are covered with a thick but smooth cuticle; the anticlinal walls are straight. Stomata are absent; trichomes similar to those of the leaf are present. At the base many trichomes are much longer. Their dimensions are for *M. stipulosa*, 20–50 μ long, with bases 10–20 μ wide—those at the base measuring up to 450 μ long, and for *M. ciliata*, 25–75 μ long with the bases 15–20 μ wide—those at the base of the petioles measuring up to 560 μ long.

Stipules

The upper epidermis consists of a single layer of polygonal cells having thin walls covered with a thin smooth cuticle; anticlinal walls sinuous; stomata and trichomes absent. The mesophyll is non-differentiated and consists of thin-walled rounded to elongated cells with large air spaces. The veins consist of a few liquified xylem elements, spirally and annularly thickened vessels and reticulately thickened tracheidal vessels and a small group of phloem tissue, the entire bundle being surmounted both above and below by thick-walled parenchyma and small groups of collenchyma. Cluster crystals of calcium oxalate similar in size to those in the leaf are present in some of the parenchymatous cells surrounding the bundles.

The lower epidermis consists of a single layer of polygonal cells having thin walls covered with a thin cuticle. In *M. stipulosa* the cuticle is ridged and in *M. ciliata* it is smooth so that no striations are visible. The anticlinal walls are straight. Paracytic stomata and trichomes similar to those on the leaf are present. In the basal part of the stipule the trichomes are numerous and measure up to 800 μ in length; a few are less than 250 μ. Along the margins there are trichomes of from 40–140 μ in length (Fig. 6, B, C, D and E). The cylindrical or slightly conical protuberances measure from 750–1850 μ long and 200–400 μ wide at the base. They have an epidermis of radially elongated cells which appear slightly

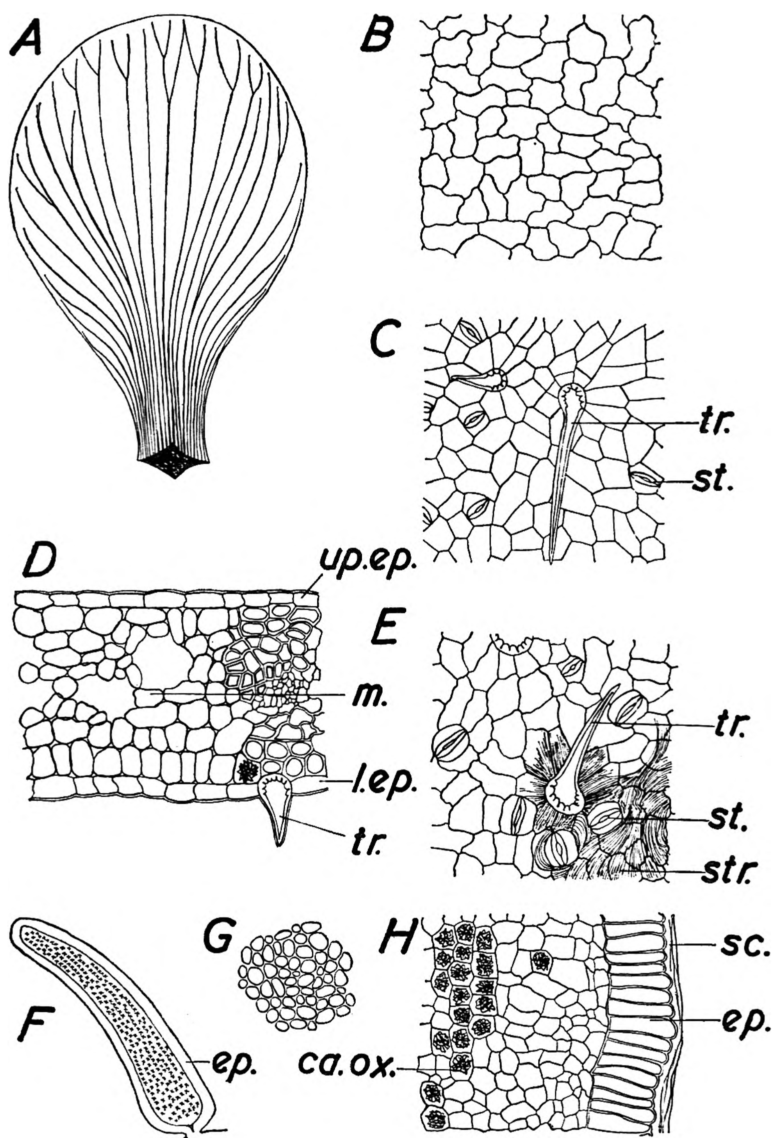

FIG. 6. A, stipule of *M. stipulosa* or *M. ciliata* × 1; B, *M. stipulosa*, stipule, upper epidermis, surface view × 160; C, *M. ciliata*, stipule, lower epidermis, surface view × 160; D, *M. ciliata*, t.s., stipule × 160; E, *M. stipulosa*, stipule, lower epidermis, surface view × 160. F, *M. stipulosa*, stipule, secretory protuberance × 25; G, epidermis, secretory protuberance, surface view × 160; H, t.s., secretory protuberance × 160. e.p., epidermal cells; l.ep., lower epidermis; m., meosphyll; s.c., secretion; st., stoma; str., striations; tr., trichome; up.ep., upper epidermis, × represents crystals of calcium oxalate.

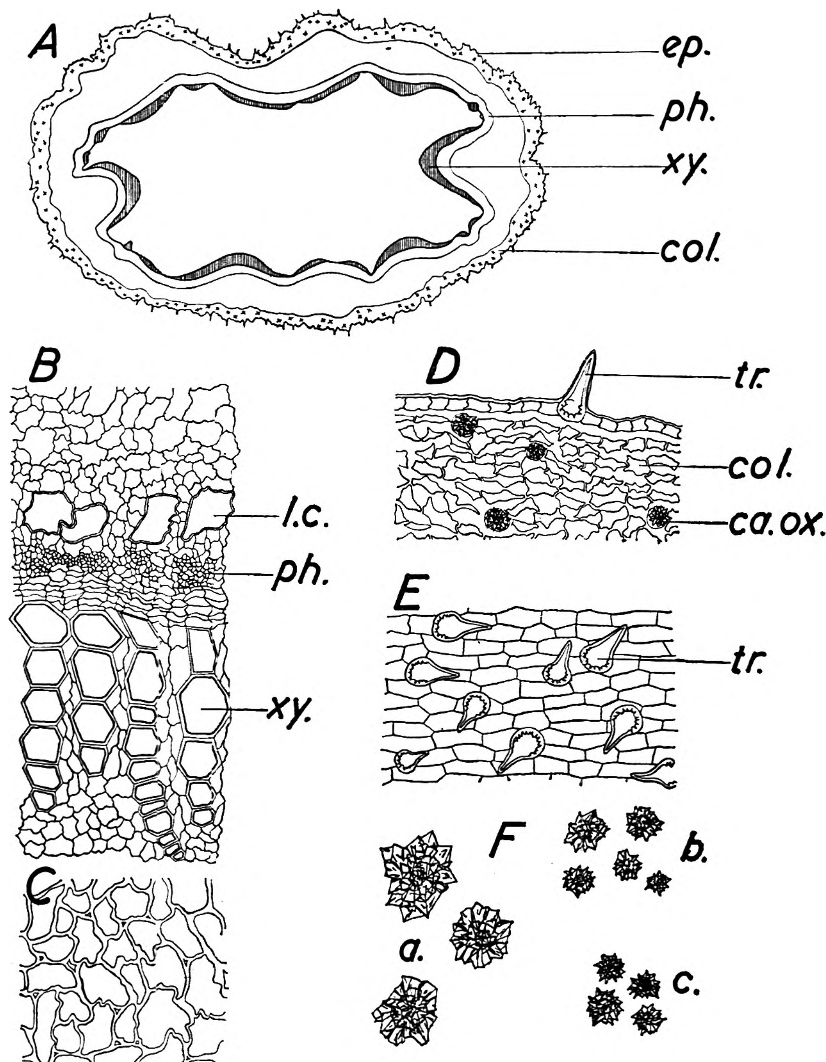

FIG. 7. *M. stipulosa*, A, t.s., young stem × 10; B, stele × 160; C, pith × 160; D, t.s., epidermal region × 160; E, young stem, epidermis, surface view × 160; F, cluster crystals of calcium oxalate × 240; from young stem of (*a*) *M. ciliata* and (*b*) *M. stipulosa*. (*c*) from leaf of *M. stipulosa*. ca.ox., calcium oxalate; col. collenchyma; ep., epidermis; l.c., latex cell; ph., phloem (sieve tissue); tr., trichome; xy., xylem. X represents a crystal of calcium oxalate.

papillose when the surface is viewed obliquely. The medulla consists of thin-walled isodiametric parenchymatous cells and contains numerous cluster crystals of calcium oxalate arranged in longitudinal files of up to 18 crystals. The crystals measure from 4 to 15 μ in diameter (Fig. 6, F, G, H).

Young Stem

The anatomy of the young stem is similar to that of the petiole (Fig. 7). The crystals of calcium oxalate in some of the collenchyma are much larger, being 15–45 μ in diameter in *M. stipulosa*, and 25–65 μ in diameter in *M. ciliata*. The latex cells in the outermost part of the phloem are much larger than those in the midrib or petiole, their maximum diameter being 100 μ for *M. stipulosa*, and 240 μ for *M. ciliata*. The pith is similar to that in the petiole. No starch or calcium oxalate is present in the phloem, xylem or pith.

Discussion

The similarity of the anatomical structure of the leaves, stipules and young stem of these two species supports the morphological evidence that *M. stipulosa* and *M. ciliata* are closely related. Apart from minor differences, e.g., the maximum recorded sizes of the calcium oxalate crystals and trichomes and the appearance of the cuticle on the leaf surface, the only significant difference between them is the structure of the lamina. The leaf of *M. stipulosa* is typical of a shade leaf, while that of *M. ciliata* is typical of a sun leaf.

Two possibilities exist, the first that the leaf structure is variable and dependent upon the actual habitat of the plant, the second, that the leaf structure has become permanently established and is independent of the habitat. Nordhausen (1903, 1912) has shown that for some trees, e.g., oak, beech, the type of leaf is predetermined by conditions of former vegetative growth and that exposed trees, transplanted in the shade will continue to show sun leaves. Other workers, however, found that the shade leaves of ivy could be changed to sun leaves by exposure to bright light.

It may well be that the two species described are geographical variants which have now become established as distinct species. This is supported not only by the slight morphological and anatomical differences and by the different alkaloidal spectrum (Beckett, Shellard and Tackie, 1963) but also by the fact that these particular samples of *M. cilata* leaf were taken from trees growing in the shade and of *M. stipulosa* leaf from trees fully exposed to the sun.

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The paper was presented by DR. SHELLARD.

Short Communication

THE STRUCTURE OF CASCAROSIDES A AND B

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A PREVIOUS communication (Fairbairn and Simic, 1960) reported the isolation and properties of two substances, cascaroside A and cascaroside B, obtained from Cascara (*Rhamnus purshiana* DC bark). Their general properties, ultra-violet light absorption curves and behaviour on chromatograms were similar, but a significant difference was noted in their optical rotations. The further work reported in this present paper is concerned with the relationship between cascarosides A and B, the products of hydrolysis other than barbaloin and the position of attachment of these groups to barbaloin.

The Relationship between Cascaroside A and B

Since the infra-red spectra of cascarosides A and B were almost identical, it seemed likely that they were stereoisomeric since no significant structural differences could be deduced from the curves. The nature of the isomerism was established by acetylation of the two cascarosides with acetic anhydride in pyridine when two anthranol acetates were obtained which were identical in optical rotation ($[\alpha]_D^{20°} = -51°$) and melting point with no depression of the latter on admixture. They can be represented by part structure (II) in which the tetrahedral arrangement at C_{10} responsible for the isomerism of the cascarosides has now become planar.

R_1O O OR_2 — CH_2OH — H $C_6H_{11}O_5$

I. *Cascarosides*
R_1, R_2 = H or glycone

R_3O OAc OR_4 — CH_2OAc — $C_6H_7O(OAc)_4$

II. *Acetyl cascaroside*
R_3, R_4 = acetyl or acetyl glycone

The isomerism of the cascarosides is therefore attributable to the aglycone moiety and not to the nature or position of attachment of the glycones. This was confirmed by hydrolysis of cascarosides A and B in N hydrochloric acid at 70° under nitrogen when two different barbaloins (the aglycones) were obtained. Their melting points, R_F values in three different systems and reactions to the usual barbaloin tests (including the cupraloin test) were identical. They differed slightly in crystalline form; aglycone A readily formed yellow needles whilst aglycone B crystallised with difficulty and tended to form clusters of fine needles. The main

difference however was in optical rotation: the $[\alpha]_D^{20}$ in methanol (c, 2·5) for aglycone A was $+7{\cdot}60°$ and for aglycone B was $-47{\cdot}8°$. Cascaroside A is therefore a glycoside of (+)-barbaloin and cascaroside B of (−)-barbaloin. These two isomers of barbaloin may be barbaloin and iso-barbaloin respectively which, Mühlemann (1952) suggests, are possibly optical isomers.

The Glycones

Glucose was shown to be present in the hydrolysate of both cascarosides. It was identified by its R_F value and by its infra-red spectrum. Quantitatively, the cascarosides yielded 56 to 60 per cent barbaloin and about 40 per cent by weight of glycone. Of this only about half was glucose; the rest was a non-reducing glycone. The presence of two glycones was confirmed by methanolysis of the cascarosides using methanol in the presence of ZeoKarb 225^+ resin. Paper chromatography showed 1-α-methyl-D-glucose ($R_F = 0{\cdot}17$) and a second non-reducing component ($R_F = 0{\cdot}03$) revealed as a yellow spot on exposure to iodine vapour.

Both cascarosides undergo oxidation with aqueous sodium periodate at 0° with the consumption of 7 moles of reagent. Under these conditions barbaloin (and homonataloin, Haynes, Henderson and Tyler, 1960) requires 2 moles; hence the two glycones must consume 5 moles of periodate. If we assume that the glucose is attached to the barbaloin by a 1-glucosidal link (indicated by rapid acid or enzyme hydrolysis) then it will consume 2 moles of periodate, leaving 3 moles for the other glycone. This would then contain the part structure $(CHOH)_4$ and would probably be linked to the barbaloin by means of a further –C–O– group. Such a 5 carbon molecule would fit our previous analytical results which indicate that the proportion of this glycone present is less than that of glucose; that is, the molecular weight is less than 180.

Attachment of the Glycones to the Barbaloins

Since both cascarosides have identical acetyl derivatives, the points of attachment and conformation of the glycones must be similar in the two compounds. One possibility is that one glycone is attached to each of the two phenolic groups of the barbaloin and in this connection certain ultra-violet spectra are of interest. Hydrolysis of the cascarosides to barbaloin shifts the absorption maximum at 325 mμ to 354 mμ; conversely methylation of barbaloin to dimethyl-barbaloin (I; $R_1 = R_2 =$ Me) shifts the maximum back to 325 mμ. Although this agrees with the suggestion that cascarosides, like dimethyl-barbaloin, have no free phenolic groups, their infra-red spectra indicate that both phenols are not blocked and that the shifts observed in ultra-violet light are probably due to phenomena more complex than phenol-ketone chelation. Thus the carbonyl group in the cascarosides, as in barbaloin, absorbs at 1636 cm.$^{-1}$ which is about 40 cm.$^{-1}$ lower than the unbonded quinone absorption. Since the carbonyl group in barbaloin is clearly bonded with its two free phenolic groups (cf. Haynes, Henderson and Taylor, 1960) it seems probable that the cascarosides contain at least one free phenolic

-OH group with which the carbonyl group is bonded. This is supported by the following observations:

(i) The ultra-violet absorption spectrum in alkaline solution produces a shift from 320 to 390 mμ. (In barbaloin the shift is from 354 to 388 mμ).

(ii) Both barbaloin and cascaroside in ethanolic solution give a deep green colour with ferric chloride (however sennoside, rheinanthrone-8-glucoside and rheinanthrone do not give colours with this reagent).

(iii) Both cascarosides and barbaloin give a yellow complex with methanolic magnesium acetate in which the peaks at 323 and 354 mμ have been shifted to 394 and 430 mμ respectively. The corresponding peaks in rheinanthrone glucoside and rheinanthrone shift from 340 to 380 and from 364 to 450 mμ respectively.

The spectroscopic results therefore indicate that there is, at least, one free phenolic group present. The two glycones may either be linked to each other and attached to one of the phenolic groups or one glycone may be so attached and the other attached to one of the alcoholic groups of the barbaloin.

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A NEW METHOD OF STREPTOMYCIN CHROMATOGRAPHY AND ITS USE IN THE EXAMINATION OF THE REACTION BETWEEN STREPTOMYCIN AND AMMONIA

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A new paper chromatographic method for streptomycin and similar compounds is described. It is used to detect the products of reaction between streptomycin and ammonia in low purity streptomycin; the presence of such compounds causes the microbiological and maltol methods of streptomycin assay to give different results.

A NUMBER of solvent systems have been described for the paper chromatography of streptomycin and that of Winsten and Eigen (1948) appears to have been given the greatest attention. Its use, however, suffers from "double spotting" caused by the salt content of the sample (Consden, 1944; Peterson and Reineke, 1950). The present paper describes a solvent system which is unaffected by salt content. It is used to examine the reaction between streptomycin and ammonia first mentioned by Solomons and Regna (1950).

EXPERIMENTAL

Qualitative Chromatography

Solvent system. Following the use of pentachlorophenol for the extraction of streptomycin-like compounds (Yabuta, 1955), we find that the solvent system of Winsten and Eigen (1948) is best replaced as follows: Sodium hydroxide (15 g.) in water (150 ml.) is shaken for 1 hr. with pentachlorophenol (30 g.) and n-butanol (850 ml.). Any lower aqueous phase is discarded and the upper phase filtered through glass wool.

Whatman No. 4 paper buffered to pH 7·0 with 2 per cent phosphate buffer was used. Sample aliquots within the range 10 to 800 units streptomycin were placed on the paper and developed for about 18 hr. (descending technique). This produced a movement of the streptomycin zone of about 12 in. Equilibration of the papers in the chromatography tank before development was not necessary. The papers were then washed by drawing them through ether to remove the pentachlorophenol, and so assist detection. Two methods of detection were used: (i) The papers were dipped in 0·005M periodic acid and then in 0·01M benzidine, both solutions being in acetone, the components thus being revealed as yellow or white spots on a blue background (Gordon, 1956). (ii) This comprised dipping the papers in a mixture of diacetyl, alkali and α-naphthol (Foster and Ashton, 1953) which showed streptomycin and associated compounds as red spots on a white background. Both reagents were sensitive to as little as 10 μg. streptomycin.

Quantitative Chromatography

For an initial separation 600–900 units streptomycin were applied to a sheet of Whatman's No. 4 paper and chromatographed as above. The required zones, indicated by tracer strips treated with one of the colorimetric indicators, were cut out. These were then macerated with 10 ml. of pH 4·4 McIlvaine buffer to extract the streptomycin, after which the solution was filtered and assayed by a sensitive method already described by Savitskaya and Kartseva (1953).

This consists in forming the 2,4-dinitrophenylhydrazone in aqueous solution and in measuring the extinction of this at 430 mμ. The method was used satisfactorily with streptomycin concentrations of 10–150 μg./ml. and about ten equivalents of the reagent. Formation of the 2,4-dinitrophenylhydrazone by heating in a boiling water-bath for 2½ min. gave results similar to those obtained by standing at room temperature for 2 hr., as recommended by Savitskaya and Kartseva, or by standing for periods up to 48 hr. The shorter reaction time at high temperature was therefore adopted. Excess reagent was removed by washing with n-butyl acetate. This caused no measurable loss of 2,4-dinitrophenylhydrazone, but several washings were required to rid the solution of excess reagent and give a constant extinction value which was measured at 430 mμ.

Streptomycin of known purity was used as a standard and treated similarly. Tests with solutions of pure streptomycin showed an error of about ±3 per cent between replicates.

Electrophoresis. This was carried out using Shandon apparatus and Whatman 3MM paper with a buffer of M/15 pH 5·6 potassium phosphate (Foster and Ashton, 1953).

Other analyses. Microbiological assays were carried out using the cavity plate method described by Brownlee and his co-workers (1948) with *Bacillus subtilis* NCTC 8236 as test organism. The method described by Grove and Randall (1955) was used for maltol assays.

Results and Discussion

The Reaction Between Streptomycin and Ammonia

The reaction between streptomycin and ammonia yields bis-(α-hydroxystreptomycylamine) (IV) a highly toxic substance with a high maltol/microbiological streptomycin ratio (Solomons and Regna, 1950). These authors showed that in aqueous solution at pH 2·5 it reverts to streptomycin (I) in a few hours, and that upon hydrogenation at a platinum catalyst it produces streptomycylamine (II) and dihydrostreptomycin (III).

We find that bis-(α-hydroxystreptomycylamine) (IV) has an R_F value of 0·08 relative to streptomycin as 1, when chromatographed under the conditions described above. Its electrophoretic movement towards the cathode is about 0·3 relative to streptomycin as 1. The hydrogenation products streptomycylamine and dihydrostreptomycin have R_F values of 0 and 0·75 respectively. These values were checked by comparison with streptomycylamine prepared by the reduction of streptomycin

oxime and with dihydrostreptomycin prepared by hydrogenation of streptomycin. Both moved towards the cathode on electrophoresis giving values of 0·66 (streptomycylamine) and 1·0 (dihydrostreptomycin) relative to streptomycin as 1.

St–CHO (streptomycin) (I)

NH_3 ↙ ↘ NH_3

St–CH(OH)(NH_2) (V)

St–CH(OH)–NH–CH(OH)–St (IV) $\xrightarrow{H_2}$ St–CH_2NH_2 (II) + St–CH_2OH (III)

Mixtures of streptomycin sulphate and ammonium sulphate gave rise to three main components on chromatography: two major zones at R_F values 0·08 and 0·48 with a minor zone at R_F 0·23. The component of R_F 0·08 corresponds with bis-(α-hydroxystreptomyclamine) and the R_F 0·48 component is probably streptomycin monoaldehyde ammonia (V) because it is also produced by acid hydrolysis of bis-(α-hydroxystreptomycylamine). The zone at R_F 0·23 has not been identified.

The reaction between ammonium sulphate and streptomycin was examined for its effect on the maltol and microbiological assay methods. A solution of streptomycin containing 10 per cent by weight of ammonium sulphate, based on the weight of streptomycin, was made and portions of the solution were adjusted separately to pH 7·0 and pH 9·0. No reduction in the result from the maltol or microbiological assay over control values was observed in the experiment at pH 7·0. This suggests that no reaction had taken place between the streptomycin and ammonium sulphate. The experiment at pH 9·0 showed no reduction in the maltol assay but a loss of about 6 per cent of the microbiological activity after 2 hr., indicating that some reaction had now taken place.

The Examination of Low Purity Streptomycin

Streptomycin of less than 90 per cent purity nearly always gave a substantially higher assay result by the maltol or 2,4-dinitrophenylhydrazine methods than by the microbiological method, the difference often being as much as 10 per cent. Impure streptomycin was found to contain small proportions of the components of R_F 0·08, 0·48 and 0·23 shown above to result from the reaction of streptomycin with ammonia. Furthermore, when an eluate of the component of R_F 0·08, bis-(α-hydroxystreptomycylamine) (IV) was allowed to stand overnight in solution at pH 2·5 it gave some streptomycin and also some of the component of R_F 0·48 (streptomycin monoaldehyde ammonia, V) which did not further readily change to streptomycin. The presence of bis-(α-hydroxystreptomycylamine) in impure streptomycin was confirmed by electrophoresis.

The reactivities of these components with various indicators is shown in Table I, whilst the quantitative examination of several samples of

impure streptomycin is shown in Table II. The results in Table II illustrate the interference of the R_F 0·08 and 0·48 components with the maltol assay and show the relative specificity of the microbiological method for streptomycin.

TABLE I

DETECTION OF IMPURITIES IN LOW PURITY STREPTOMYCIN BY CHROMATOGRAPHY AND VARIOUS INDICATORS

Relative R_F values	Known or probable identity	Colour produced by indicator				
		Elson Morgan	Sakaguchi	Diacetyl	Periodate/ benzidine	Silver nitrate
0·08	Bis-(α-hydroxystreptomycyl-amine)	Blue	Pink	Pink	Yellow	—
0·23	?	—	—	Pink	White	—
0·48	Streptomycin monoaldehyde ammonia	Blue	Pink	Pink	White	—
1·00	Streptomycin	Blue	Pink	Pink	Yellow	Brown

Eluates of the R_F 0·08 and 0·48 component were next given an ion-exchange treatment to remove phosphate and then assayed by the maltol and microbiological methods. The maltol to microbiological assay ratios were found to be 3·2 and 1·3 for the respective components, showing them to be principal causes of the differences between the assay results by the two methods.

TABLE II

COMPARISON OF ASSAYS ON LOW POTENCY STREPTOMYCIN SULPHATE

Sample	Streptomycin (units/mg.)					
	Chromatographic assay					
	Bis-(α-hydroxy-streptomycylamine) R_F 0·08	Streptomycin monoaldehyde ammonia R_F 0·48	Streptomycin R_F 1·00	Total	Maltol assay	Microbiological assay
A	40	40	640	720	680	630
B	10	10	640	660	690	640
C	30	40	650	720	700	650
D	40	20	570	630	650	560
E	70	40	560	670	640	570
F	50	30	580	660	660	610

Note.—Pure streptomycin sulphate has a potency of 798 units/mg.

Hydrogenation of a solution of impure streptomycin often led to a rapid initial uptake of hydrogen although the reaction proceeded very slowly in the final stages. A long time was taken to lower the residual streptomycin, as measured by maltol assay, below about 3 per cent. The solution thus appeared to contain an impurity, representing up to about 3 per cent of the streptomycin, which assayed as streptomycin by the maltol method and which was not very readily reduced. Similar results had been reported by Kaplan, Fardig and Hooper (1954) who also attributed reduction difficulties to the presence of maltol-producing

impurities. Chromatographic examination of the course of hydrogenation showed the production of a component of R_F 0, probably streptmycylamine; electrophoretic examination confirmed this supposition.

Pereira (1961), in studying ion-exchange chromatography of streptomycin, had shown the presence of unidentified maltol-producing impurities at R_F 0·18, 0·32 and 0·58 relative to streptomycin as 1, using the Winsten and Eigen (1948) solvent system. These impurities had a maltol to microbiological assay ratio of 2·5, 1·9 and 1·5 respectively. They may well have been similar to those found in this present study or similar to those described by Winsten and Eigen (1948).

A comparison of the chromatographic and electrophoretic results on impure streptomycin and on the reaction products of streptomycin and ammonia is given in Table III.

TABLE III

CHROMATOGRAPHIC AND ELECTROPHORETIC SEPARATION OF LOW PURITY STREPTOMYCIN AND OF THE PRODUCTS OF THE STREPTOMYCIN/AMMONIA REACTION

Known or proposed identity	Movement relative to streptomycin = 1		Occurrence
	Chromatography	Electrophoresis	
Streptomycylamine	0	0·66	Found in hydrogenated bis-(α-hydroxystreptomycylamine) and hydrogenated impure streptomycin
Bis-(α-hydroxystreptomycylamine)	0·08	0·33	Produced by the streptomycin/ammonia reaction and found in impure streptomycin
?	0·23	—	„
Streptomycin monoaldehyde ammonia	0·48	—	„
Dihydrostreptomycin ..	0·75	1·00	Found in hydrogenated bis-(α-hydroxystreptomycylamine) and is produced by hydrogenating streptomycin
Streptomycin	1·00	1·00	—

The various experiments described above showed the similarity between two major impurities in low purity streptomycin and the products from the reaction between streptomycin and ammonia.

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