BRITISH PHARMACEUTICAL CONFERENCE

NINETY-SIXTH ANNUAL MEETING, BOURNEMOUTH, 1959

REPORT OF PROCEEDINGS

OFFICERS:

President:

G. H. HUGHES, M.P.S.

Chairman:

H. TREVES BROWN, B.Sc., F.P.S., London.

Vice-Chairmen:

R. R. BENNETT, B.Sc., F.P.S., F.R.I.C., Eastbourne.
HAROLD DEANE, B.Sc., F.P.S., F.R.I.C., Sudbury.
H. HUMPHREYS JONES, F.P.S., F.R.I.C., Liverpool.
T. E. WALLIS, D.Sc., F.P.S., F.R.I.C., F.L.S., London.
H. BRINDLE, M.Sc., F.P.S., F.R.I.C., Manchester.
B. A. BULL, F.P.S., A.R.I.C., Nottingham.
NORMAN EVERS, Ph.D., F.R.I.C., Ware.
A. D. POWELL, M.P.S., F.R.I.C., Nottingham.
H. BERRY, B.Sc., Dip.Bact. (Lond), F.P.S., F.R.I.C., Eastbourne.
H. B. MACKIE, B.Pharm., F.P.S., Brighton.
G. R. BOYES, L.M.S.S.A., B.Sc., F.P.S., F.R.I.C., London.
H. DAVIS, C.B.E., B.Sc., Ph.D., F.P.S., F.R.I.C., London.
J. P. TODD, Ph.D., F.P.S., F.R.I.C., Glasgow.
K. BULLOCK, M.Sc., Ph.D., F.P.S., F.R.I.C., Manchester.
FRANK HARTLEY, B.Sc., Ph.D., F.P.S., F.R.I.C., London.
G. E. FOSTER, B.Sc., Ph.D., F.R.I.C., Dartford.

Honorary Treasurer:

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

Honorary General Secretaries:

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

Other Members of the Executive Committee:

THE CHAIRMAN of the Executive of the Scottish Department of the Pharmaceutical Society of Great Britain (*ex officio*).
The PRESIDENT of the Pharmaceutical Society of Ireland (*ex officio*).
The PRESIDENT of the Pharmaceutical Society of Northern Ireland (*ex officio*).
The EDITOR of the *Journal of Pharmacy and Pharmacology* (*ex officio*).
The CHAIRMAN and Honorary Secretary of the Local Committee (*ex officio*).

D. C. ADAMSON, F.R.I.C., Greenford.
*E. A. BROCKLEHURST, M.P.S., J.P., Hull.
A. W. BULL, B.Sc., B.Pharm., F.P.S., F.R.I.C., Nottingham.
J. G. DARE, Ph.D., F.P.S., Leeds.
*D. W. HUDSON, M.P.S., Hove.
W. H. LINNELL, D.Sc., Ph.D., F.P.S., F.R.I.C., London.
J. B. LLOYD, M.P.S., Manchester.
*T. REID, M.P.S., Haselmere.
J. B. STENLAKE, B.Sc., Ph.D., F.P.S., A.R.I.C., Glasgow.

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

REPORT OF PROCEEDINGS

PROCEEDINGS OF CONFERENCE

BOURNEMOUTH 1959

THE OPENING SESSION

The opening session of the Conference was held in the Pavilion ballroom in Bournemouth, on Monday, September 21, with Mr. G. H. Hughes, President of the Conference (President of the Pharmaceutical Society) in the Chair. On the platform were the Chairman of the Conference (Mr. H. Treves Brown), the Mayor of Bournemouth (Alderman L. V. Barney), the Chairman and Secretary of the Local Committee (Mr. H. Ridehalgh and Mr. D. F. Smith), the Honorary Treasurer, the Honorary General Secretaries together with members of the Conference Executive Committee.

The President introduced the Mayor of Bournemouth, who welcomed the Conference to Bournemouth. The President thanked the Mayor on behalf of the Conference for his welcome.

The President then handed over the further conduct of the Conference to the Chairman (Mr. H. Treves Brown), who delivered his address entitled "Patents in Pharmacy and Medicine," which is printed in full in the *Journal of Pharmacy and Pharmacology*, 1959, **11**, Supplement pages *9 T* to *43 T*.

On the proposition of Mr. J. C. Hanbury, seconded by Professor A. D. Macdonald, the Conference accorded a hearty vote of thanks to the Chairman for his address.

CIVIC RECEPTION

On the evening of Monday, September 21, a Civic Reception was given at the Pavilion Ballroom. The guests were received by the Mayor and Mrs. Barney and the Chairman of the Local Committee and Mrs. Ridehalgh. A dance was held after the reception.

THE SCIENCE SESSIONS

Meetings were held on Monday, Tuesday, Wednesday and Friday, September 21, 22, 23, 25 at the Town Hall and the Pavillion, the Chairman and Immediate Past Chairman presiding. During the sessions the following 36 papers were communicated:—

1. Potential Analgesics. The Stereochemistry of some Isomeric Piperidinol Derivatives. By N. J. Harper, M.Sc., Ph.D., F.P.S., A.R.I.C., A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C. and A. D. J. Balon, B.Pharm., Ph.D.
2. Neuromuscular Blocking Agents. Part IV. The Synthesis and Study of *N*- and *S*-Alkyl Variants of Dihexasulphonium and Dihexazonium Triethiodides. By Fiona Macleod Carey, B.Sc., D. Edwards, B.Sc., F.R.S., A.R.I.C., J. J. Lewis, M.Sc., F.P.S., and J. B. Stenlake, B.Sc., Ph.D., F.P.S., A.R.I.C.
3. Neuromuscular Blocking Agents. Part V. Linear *NNNN*-Tetra-onium. *NNSNN*-Penta-onium and *NNNNNN*-Hexa-onium Compounds. By D, Edwards, B.Sc., F.P.S., A.R.I.C., J. J. Lewis, M.Sc., F.P.S., J. B. Stenlake, B.Sc., Ph.D., F.P.S., A.R.I.C., and Fay Stothers, B.Sc.
4. Neuromuscular Blocking Activity in some *NS*-Bis-onium Compounds. By T. C. Muir, B.Sc., M.P.S. and J. J. Lewis, M.Sc., F.P.S.
5. The Ganglionic Blocking Activity of a Series of Tertiary Sulphonium Quaternary Ammonium Salts. By D. M. Brown, B.Sc., F.P.S. and D. H. Turner, B.Pharm., M.P.S.
6. The Spectrophotometric Determination of Certain Alkaloids and Application to Pharmaceutical Preparations. By A. H. J. Cross, B.Sc., D. McLaren, B.Sc. and S. G. E. Stevens, B.Sc., F.R.I.C.
7. A New Synthesis for 1-Aminohydantoin and Nitrofurantoin. By D. Jack, B.Sc., F.P.S., A.R.I.C.
8. A Light-Scattering Study of Lysolecithin Sols. By N. Robinson, M.Sc., Ph.D., A.R.I.C. and L. Saunders, D.Sc., Ph.D., F.R.I.C.
9. Interfacial Films between Benzene and Solutions of Salts of Arabic Acid. By E. Shotton, B.Sc., Ph.D., F.P.S., F.R.I.C. and K. Wibberley, B.Pharm., M.P.S., A.R.I.C.
10. The Size Analysis of Phenothiazine. By M. J. Thornton, B.Sc., A.R.I.C.
11. Further Studies of some Esters of 4-Aminosalicylic Acid. By D. J. Drain, B.A., F.R.I.C., R. Lazare, B.Sc., G. A. Poulter, K. Tattersall, B.Sc. and Alicjz Urbanska.
12. The Assay of Anti-haemophilic Globulin. By R. Maxwell Savage, Ph.D., M.A., F.R.I.C.

13. The Antagonism of the Antibacterial Action of Mercury Compounds. Part III. The Effects of Certain Sulphydryl Compounds on *E. coli* I. By A. M. Cook, B.Pharm., Ph.D., Dip.Bact., F.P.S., F.R.I.C. and K. J. Steel, B.Pharm., Ph.D., F.P.S.
14. The Antagonism of the Antibacterial Action of Mercury Compounds. Part IV. Qualitative Aspects of the Antagonism of the Antibacterial Action of Mercuric Chloride. By A. M. Cook, B.Pharm., Ph.D., Dip.Bact., F.P.S., F.R.I.C. and K. J. Steel, B.Pharm., Ph.D., F.P.S.
15. A Study of Factors Affecting the Inactivation of Quaternary Ammonium Compounds on Agar. By M. J. Groves, M.Pharm., M.P.S. and H. A. Turner.
16. The Comparative Protective Effects of Degraded Carrageenin and Aluminium Hydroxide on Experimentally Produced Peptic Ulceration. By W. Anderson, B.Sc., Ph.D., F.P.S. and J. Watt, M.D.
17. The Effect of Protoveratrine A on Potassium and Calcium Movements in Muscle and Nerve. By R. E. Lister, B.Sc., Ph.D. and J. J. Lewis, M.Sc., F.P.S.
18. The Effects of Protoveratrine on Plasma Potassium Levels in the Cat and Rabbit. By R. E. Lister, B.Sc., Ph.D. and J. J. Lewis, M.Sc., F.P.S.
19. Metallic Cations and the Antibacterial Action of Oxine. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C., R. N. Dar, B.Pharm., B.Sc. and Ann E. Robinson, B.Pharm., Ph.D., A.R.I.C.
20. A comparison of the Spasmolytic Effects of Two Phenylethylamines and some Observations on Morphine-like Activity. By A. McCoubrey, B.Sc., Ph.D., M.P.S., F.R.I.C.
21. Some Effects of Hydrallazine on Blood Iron and an Iron-Containing Enzyme System. By S. M. Kirpekar, B.Sc., Ph.D. and J. J. Lewis, M.Sc., F.P.S.
22. Physico-Chemical Studies of Aspirin with Glycine. By H. D. C. Rapson, B.Sc., A.R.I.C., D. O. Singleton, B.Sc., A. C. E. Stuart, M.A. and Mary P. Taylor, B.Sc.
23. The Flask Combustion Technique in Pharmaceutical Analysis. Iodine-containing Substances. By C. A. Johnson, B.Pharm., B.Sc., F.P.S., A.R.I.C. and C. Vickers, B.Sc., A.R.I.C.
24. Phytochemical Changes Initiated by Insects. Part I. Preliminary Work on Leaves and "Bean Galls" of *Salix fragilis* L. By S. B. Challen, B.Pharm., B.Sc., Ph.D., F.P.S., F.L.S.
25. Gas Chromatography in Routine Pharmaceutical Analysis. By C. B. Baines, B.Sc. and K. A. Proctor, M.Sc., F.R.I.C.
26. A Note on the Use of Membrane Filters in Sterility Testing. By G. Sykes, M.Sc., F.R.I.C. and Margaret C. Hooper, M.Sc., Dip.Bact.
27. The Preparation and Evaluation of some Phenolic Ethers as Antifungal Agents. By L. V. Coates, A.I.M.L.T., D. J. Drain, B.A., F.R.I.C., F. June Macrae, B.Sc. and K. Tattersall, B.Sc.
28. Sodium Novobiocin: Stability Aspects. By M. J. Busse, B.Pharm., M.P.S., K. A. Lees, F.P.S., D.B.A. and V. J. Vergine, B.Pharm., F.P.S.
29. "Footprints" in Adsorbents. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C. and Patricia Anderson, B.Sc. (Pharm.).
30. A Contribution to Powder Compaction Theory by the Pressing of Regular Arrangements of Spheres. By D. Train, M.C., B.Pharm., B.Sc. (Eng.), Ph.D., A.C.G.I., D.I.C., F.P.S., F.R.I.C., A.M.I.Chem.E. and J. N. Carrington, B.Pharm., Ph.D., M.P.S.
31. Pharmacological and Chemical Observations on some Toxic Nectars. By Fiona Macleod Carey, B.Sc., J. J. Lewis, M.Sc., F.P.S., J. L. MacGregor, Dip.Hort., and M. Martin-Smith, M.Sc., Ph.D.
32. Indole Derivatives in Tomatoes. By G. B. West, B.Pharm., D.Sc., Ph.D., F.P.S.
33. The Characterisation of Crystalline and Amorphous Aloin. By R. E. Lister, B.Sc., Ph.D. and R. R. A. Pride, B.Sc., F.R.I.C.
34. Some Aspects of the Storage and Testing of Sterilised Catgut. By G. R. Wilkinson, F.P.S., E. L. Robins, B.Pharm., A.R.I.C., J. J. Grimshaw, B.Sc., F.I.S., F.S.S. and S. P. A. Hudson, B.Pharm.
35. The Absorption and Distribution of Halothane. By W. A. M. Duncan, B.Sc., Ph.D.
36. An Indicator Control Device for Ethylene Oxide Sterilisation. By A. Royce, F.P.S. and C. Bowler.

The papers are printed in full with reports of discussions in the *Journal of Pharmacy and Pharmacology*, 1959, **11**, Supplement, pages *67 T–298 T.*

REPORT OF PROCEEDINGS

THE SYMPOSIUM SESSION

A symposium on "The Modification of the Duration of Drug Actions" was held on Thursday, September 24. The CHAIRMAN presided. The introductory papers were presented by Professor A. Wilson and Dr. R. P. Edkins. The meeting is reported in the *Journal of Pharmacy and Pharmacology*, 1959, **11**, Supplement, pages *44 T–66 T*.

PROFESSIONAL SESSIONS

With the President of the Conference, Mr. G. H. Hughes in the chair, professional sessions were held on the mornings of Tuesday, September 22, when Mr. H. Steinman and Mr. E. J. W. Cuer read introductory papers to the subject "The Role of Local Organisations in the Profession of Pharmacy", and Friday, September 25, when Dr. F. Wrigley and Mr. W. K. Fitch read introductory papers to the subject "The Advertising of Medicines". Full reports of the papers and discussions were published in *The Pharmaceutical Journal*, 1959, **183**, 166–169, 183–186, 207–211, 234–238, 259–261.

THE CLOSING SESSION

The closing session of the Conference was held on Friday, September 21, in the Pavilion Theatre, the Chairman presiding.

VOTE OF THANKS TO THE LOCAL COMMITTEE

The Chairman called on Mr. S. Hughan to propose a vote of thanks to the Local Committee. This was seconded by Miss D. M. Jones. Mr. Ridehalgh (Chairman of the Local Committee) replied to the vote of thanks. The Chairman then presented to the Bournemouth and District Branch an enscribed gavel provided by the Bell and Hills Fund. Mr. D. F. Smith (Chairman of the Branch) accepted and acknowledged the gift on behalf of the Branch.

ANNUAL REPORT

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

MEMBERSHIP.—In addition to Members, Honorary Members and Students of the Pharmaceutical Society, together with the Members of the Pharmaceutical Society of Northern Ireland, the Conference includes 73 members elected by the Executive who are not members of these Societies.

REPORTS ON 1958 MEETING.—The report of the meeting of the Conference at Llandudno 1958 together with the science papers and discussions were published in the tenth volume of the *Journal of Pharmacy and Pharmacology*. The papers and discussions at the Professional Sessions were published in the *Pharmaceutical Journal*, Volume 181.

CONFERENCE PAPERS 1959.—Forty-three research papers were submitted. Twenty-eight full papers and eight short communications were accepted for presentation. The Executive thanks the authors of these papers and also the authors of the papers presented to the Symposium and the Professional Sessions for their contributions. The Executive is grateful to the Editor of the *Journal of Pharmacy and Pharmacology* and to the Editor of the *Pharmaceutical Journal* for making galley proofs of the papers available before this meeting.

JOURNAL OF PHARMACY AND PHARMACOLOGY.—The Executive has been represented on the Editorial Board by the Chairman, 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 Newcastle during the week commencing September 5, 1960. On behalf of the Conference, the Executive has provisionally accepted invitations to visit Portsmouth in 1961, Liverpool in 1962 and London in 1963 and the Executive is grateful for these invitations.

OFFICERS AND EXECUTIVE OF THE CONFERENCE.—Your Executive has nominated the following Officers and Members of the Executive for 1959–1960:—

Chairman: W. H. Linnell, Vice-Chairmen: R. R. Bennett, H. Deane, H. Humphreys Jones, T. E. Wallis, H. Brindle, B. A. Bull, Norman Evers, A. D. Powell, H. Berry, H. B. Mackie, G. R. Boyes, H. Davis, J. P. Todd, K. Bullock, F. Hartley, G. E. Foster and H. Treves Brown. *Honorary Treasurer:* H. G. Rolfe. *Honorary General Secretaries:* E. F. Hersant and D. Train. Other members of the Executive: D. C. M. Adamson, A. W. Bull, J. G. Dare, G. F. Somers, J. B. Stenlake, W. T. Wing.

The above persons together with the President of the Conference (the President of the Pharmaceutical Society of Great Britain *ex officio*), the three persons nominated by the Council of the Pharmaceutical Society of Great Britain, namely the persons for the time being holding the office of Vice-President, immediate past President and one other, 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 1959–1960.

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

Dr. A. H. BECKETT proposed the acceptance of the report and the election of officers of the Conference for the ensuing year. Mr. J. W. Hadgraft seconded.

Professor W. H. LINNELL thanked the Conference on behalf of the newly-elected officers.

TREASURER'S REPORT

Mr. H. G. ROLFE presented and proposed the adoption of the following Report and Statement of Accounts for the year 1959:—

In presenting the accounts for the year ended December 31, 1958, there is one item to which I should like to call attention.

Since the Conference is now paying income tax on its investment income, as explained by the Treasurer last year, it was decided to invest as much as was considered prudent of the money in the Post Office and at the Bank in a short dated Government stock, namely 3 per cent Exchequer Stock, 1962–63, in order to obtain some capital appreciation without tying up capital for too long a period.

The Local Committee Fund of £250 having been repaid by the Llandudno Local Committee, was loaned to the Bournemouth Local Committee in November, 1958, in respect of the 1959 Conference.

The subscriptions from elected members, including a composition fee from the Pharmaceutical Society of Northern Ireland, amounted to £161 10*s.* 0*d.* and were credited to the account of the *Journal of Pharmacy and Pharmacology*.

H. G. ROLFE,
Honorary Treasurer.

BRITISH PHARMACEUTICAL CONFERENCE ACCOUNT

INCOME AND EXPENDITURE ACCOUNT, 1958

Expenditure	£	*s.*	*d.*	*Income*	£	*s.*	*d.*
Gavel—memento to Anglesey, N. Caernarvonshire and Colwyn Bay Branch	8	10	0	Income on 2½% Consols	40	5	0
Replica of Chairman's Badge, engraving, etc.	8	3	10	Interest on 3% Savings Bonds	6	0	0
Repairs to Tennis Cup	2	7	6	Interest on P.O. Savings Bank Account	9	7	9
Engraving London Challenge Cup for Bowls		10	6	Donation from Pharmaceutical Society of Northern Ireland	25	0	0
Income Tax for the year	23	12	9	Donation from Pharmaceutical Society of Ireland	25	0	0
Surplus carried to Accumulated Fund	62	8	2				
	£105	12	9		£105	12	9

BALANCE SHEET AT DECEMBER 31, 1958

Liabilities	£	*s.*	*d.*	*Assets*	£	*s.*	*d.*
Accumulated Fund. as at 31.12.57:—	1,919	16	2	Investments at cost (*a*) £1,610 2½% Consols (Donation by the late Alderman Clayton of Birmingham)	1,250	0	0
Add: Surplus 1958	62	8	2	(*b*) £200 3% Savings Bonds 1960–70	200	0	0
	1,982	4	4	(*c*) £500 3% Exchequer Stock 1962–63 (Total market value at 31st December, 1958: £1,471)	473	4	10
Income Tax due	13	15	3	Stock of Replicas (1) of Chairman's Badge	6	17	6
Local Committee Fund:— Donation from London Committee, 1953	250	0	0	Loan to Bournemouth Local Committee	250	0	0
				Post Office Savings Bank Account	14	14	3
				Cash at Westminster Bank	51	3	0
	£2,245	19	7		£2,245	19	7

Audited and found correct
T. HESELTINE
J. C. HANBURY
June 15, 1959

The President seconded, and the Report was adopted.

The Chairman then made a presentation to Mr. H. G. Rolfe, in recognition of his services as Honorary General Secretary for eleven years. Mr. Rolfe expressed his deep appreciation of the gift and the generosity of the members.

REPORT OF PROCEEDINGS

BRITISH PHARMACEUTICAL CONFERENCE

INAUGURAL MEETING HELD AT NEWCASTLE-ON-TYNE IN 1863

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

Years	*Places of Meeting*	*Chairmen*	*Local Secretaries*
1923	LONDON ..	F. W. GAMBLE.	W. J. U. WOOLCOCK, C.B.E.
1924	BATH	EDMUND WHITE, B.Sc., F.I.C.	P. J. THOMPSON. W. H. HALLETT.
1925	GLASGOW ..	EDMUND WHITE, B.Sc., F.I.C.	P. M. DUFF.
1926	LEICESTER ..	D. LLOYD HOWARD, J.P.	J. BARKER.
1927	BRIGHTON ..	D. LLOYD HOWARD, J.P.	F. W. BURGESS.
1928	CHELTENHAM ..	R. R. BENNETT, B.Sc., F.R.I.C.	P. JAMES.
1929	DUBLIN ..	R. R. BENNETT, B.Sc., F.R.I.C.	V. E. HANNA.

Years	*Places of Meeting*	*Chairmen*	*Local Secretaries*
1930	CARDIFF ..	J. T. HUMPHREY.	J. MURRAY.
1931	MANCHESTER ..	J. H. FRANKLIN.	R. G. EDWARDS.
1932	ABERDEEN ..	H. SKINNER.	H. M. DUGAN.
1933	LONDON ..	C. H. HAMPSHIRE, M.B., B.S., B.Sc., F.R.I.C.	H. N. LINSTEAD.
1934	LEEDS ..	C. H. HAMPSHIRE, M.B., B.S., B.Sc., F.R.I.C.	G. C. CRUMMACK. J. F. SIMON.
1935	BELFAST ..	F. W. CROSSLEY-HOLLAND, L.M.S.S.A.	D. L. KIRKPATRICK.
1936	BOURNEMOUTH	HAROLD DEANE, B.Sc., F.R.I.C.	V. J. SCAMPTON.
1937	LIVERPOOL ..	T. EDWARD LESCHER, O.B.E.	W. E. HUMPHREYS.
1938	EDINBURGH ..	J. RUTHERFORD HILL, O.B.E.	C. G. DRUMMOND.
1939	BIRMINGHAM ..	J. RUTHERFORD HILL, O.B.E.	D. J. RUSHTON.
1940	LONDON ..	H. HUMPHREYS JONES, F.R.I.C.	———
1941	LONDON ..	A. R. MELHUISH.	———
1942	LONDON ..	T. E. WALLIS, D.Sc., F.R.I.C., F.L.S.	———
1943	LONDON ..	T. E. WALLIS, D.Sc., F.R.I.C., F.L.S.	———
1944	LONDON ..	H. BRINDLE, B.Sc., F.R.I.C.	———
1945	LONDON ..	H. BRINDLE, B.Sc., F.R.I.C.	———
1946	LONDON ..	B. A. BULL, A.R.I.C.	———
1947	TORQUAY ..	B. A. BULL, A.R.I.C.	T. D. EVANS.
1948	BRIGHTON ..	NORMAN EVERS, PH.D., F.R.I.C.	A. WILSON.
1949	BLACKPOOL ..	NORMAN EVERS, PH.D., F.R.I.C.	P. VARLEY. T. A. DURKIN.
1950	GLASGOW ..	A. D. POWELL, F.R.I.C.	A. OFFICER.
1951	HARROGATE ..	H. BERRY, B.Sc., Dip. Bact. (London), F.R.I.C.	R. W. JACKSON.
1952	NOTTINGHAM ..	H. B. MACKIE, B.Pharm.	W. E. NEWBOLD. Miss G. M. WATSON.
1953	LONDON ..	G. R. BOYES, L.M.S.S.A., B.Sc., F.R.I.C.	J. M. ROWSON.
1954	OXFORD ..	H. DAVIS, C.B.E., B.Sc., PH.D., F.R.I.C.	T. R. HARDY.
1955	ABERDEEN ..	J. P. TODD, PH.D., F.R.I.C.	G. L. DICKIE.
1956	DUBLIN ..	K. BULLOCK, M.Sc., PH.D., F.R.I.C.	D. J. KENNELLY.
1957	BRISTOL ..	F. HARTLEY, B.Sc., PH.D., F.R.I.C.	E. GEORGE.
1958	LLANDUDNO ..	G. E. FOSTER, B.Sc., PH.D., F.R.I.C.	M. H. THOMAS.
1959	BOURNEMOUTH	H. TREVES BROWN, B.Sc., F.P.S.	D. F. SMITH.

Honorary Treasurers (One)

1863 to 1870, H. B. BRADY, F.R.S.
1870 to 1877, GEORGE F. SCHACHT, F.C.S.
1877 to 1884, C. EKIN, F.C.S.
1884 to 1888, C. UMNEY, F.I.C., F.C.S.
1888 to 1890, W. MARTINDALE, F.C.S.
1890 to 1893, R. H. DAVIES, F.I.C., F.C.S.
1893 to 1898, JOHN MOSS, F.I.C., F.C.S.
1898 to 1912, JOHN C. UMNEY, F.C.S.
1912 to 1925, D. LLOYD HOWARD, J.P., F.C.S.
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. MARNS.
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 H. G. ROLFE, B.Sc., F.R.I.C.

(*Honorary General Secretaries (Two)*

1863 to 1880, PROF. ATTFIELD, PH.D., F.R.S.
1863 to 1871, RICHARD REYNOLDS, F.C.S.
1871 to 1884, F. BENGER, F.C.S.
1880 to 1882, M. CARTEIGHE, F.C.S.
1881 to 1886, SIDNEY PLOWMAN, F.R.C.S.
1884 to 1890, JOHN C. THRESH, M.B., D.Sc.
1886 to 1901, W. A. H. NAYLOR, F.I.C., F.C.S.
1890 to 1903, F. RANSOM, F.C.S.
1903 to 1909, EDMUND WHITE, B.Sc., F.I.C.
1901 to 1921, E. SAVILLE PECK, M.A.
1909 to 1919, HORACE 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.

Closing Session (continued)

PLACE OF MEETING FOR 1960

Mr. T. D. CLARKE on behalf of the Newcastle District and Northumberland Branch, extended an invitation to hold the Conference in Newcastle in 1960.

Mr. A. G. M. MADGE proposed that the invitation be accepted, and Mr. G. J. W. FERREY seconded. The vote was put to the meeting and unanimously carried.

VOTE OF THANKS TO CHAIRMAN

Mr. H. D. C. RAPSON proposed a vote of thanks to the Chairman.

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

Mr. TREVES BROWN briefly responded.

REPORT OF PROCEEDINGS

BRITISH PHARMACEUTICAL CONFERENCE

CONSTITUTION AND RULES

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

2. The Conference shall consist of:—

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

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

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

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

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

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

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

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

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

BRITISH PHARMACEUTICAL CONFERENCE BOURNEMOUTH, 1959

Chairman: H. TREVES BROWN

CHAIRMAN'S ADDRESS

PATENTS IN PHARMACY AND MEDICINE

IN accordance with its Constitution the British Pharmaceutical Conference meets annually for the discussion of matters relative to the science of Pharmacy and to further the objects of the Pharmaceutical Society. One of these objects is the advancement of Chemistry and Pharmacy. Even in these days which have seen the establishment of a Restrictive Practices Court and a Monopolies Commission it is generally accepted, perhaps more often axiomatically than after full consideration, that a patent system is an essential prerequisite of industrial research by making it possible to obtain the financial resources needed to conduct it. At no previous time in history have the benefits of chemical research as reflected in the practice of medicine been so striking, and it becomes increasingly desirable that not only those who practise the profession of pharmacy but also those who are associated in any way with its practice, whether as research chemists, administrators or users of its products, should understand something of the ways in which our patent system affects it.

A learned judge once said "It is a matter of common knowledge that . . . research scientists are 'patentwise.' If they are not, they should be[1]".

Research chemists in industry are certainly brought closely into touch with the procedure for obtaining patents but I am rather doubtful if many of those here this morning would regard themselves as "patentwise". In my limited experience, many research workers in the pharmaceutical field regard the subject of patents as something that, if possible, is best left severely alone. The explanation of the difference between the learned judge's experience and my own may lie, at least in part, in the fact that the remark was made by an American judge. Nevertheless, the second part of his observation—"If they are not, they should be"—suggests that he was not too sure of his assertion. While, therefore, there will no doubt be some research workers here this morning who know from experience what is involved in filing and prosecuting patent applications, there will be other members of the Conference, including many not engaged in research, who have no such experience. I propose, therefore, to give some account of the legal requirements and the procedure for obtaining a chemical patent in this country, to describe some of the problems involved in obtaining corresponding patent protection overseas, and then to deal with some special considerations relating to patents in the fields of pharmacy and medicine in this country. I must stress that I am speaking mainly about chemical patents, which differ in some respects from the generality of patents dealt with in the text books.

I must also emphasise that in dealing in a relatively short address with such a complex legal subject it is inevitable that I shall frequently oversimplify the position. A patent lawyer would no doubt wish to qualify with "ifs" and "buts" many of the statements I shall make.

The first patent was granted in 1449 for making coloured glass for the windows of Eton and King's Colleges[2]. Addresses on patents, however, usually start with the Statute of Monopolies, 1624, which is regarded as marking the origin of the patent system in this country and indeed throughout the world, and I am following the precedent. The reason is that the economic philosophy which led to the passing of the Act is still the basis of patent law to-day and must be kept in mind when considering what the provisions of that law should be.

The object of the Statute of Monopolies was to prevent James I from filling the royal coffers at the expense of the public by the sale of monopoly rights. Its general effect was to render void all grants of monopolies for the making or selling of anything. But by Section 6 of the quaintly worded statute an exception was made of "letters patent and grants of privilege . . . of the sole working or making of any manner of new manufactures . . . to the true and first inventor or inventors of such manufactures which others at the time of making such letters patent and grants shall not use, so as also they may be not contrary to the law or mischievous to the State, by raising prices of commodities at home, or hurt of trade, or generally inconvenient". This exception was intended to encourage the introduction of new industries into the country and the encouragement of new manufacturing activities is still the object of a patent system. Section 6 of the Statute of Monopolies is still in force to-day, and it is of the utmost importance for a patent is granted only for an "invention", which is defined in the current Act, the Patents Act, 1949, as "any manner of new manufacture . . . within section six of the Statute of Monopolies and any new method or process of testing applicable to the improvement or control of manufacture". A method of testing was not patentable before 1949 and little use has so far been made of the provision in the pharmaceutical industry.

It follows from this definition that, apart from the new provision relating to testing, the first requirement for an invention to be patentable is that it shall be for a manner, or kind, of manufacture. Secondly, it must be a manner of *new* manufacture; in technical language, the invention must have novelty, by which is meant that it must not be known.

The third requirement is that there shall have been an inventive step—a patent must have what in technical language is called subject matter. Finally, it must have utility—it must be useful. I will deal with these in turn.

Requirements for Validity

Although at the time of the Statute of Monopolies the kinds of new manufacture that were contemplated were new industries, the term now includes a manufacturing process and also the product of a process. It must be emphasised that not every discovery, even when it is useful, is

patentable, and a great deal of discussion has taken place in the Courts and elsewhere to determine what is "a manner of manufacture". The discovery must relate to something tangible—in one case[3] it was said that the invention must be concerned with a "vendible product", and although this is not the whole truth and has been modified by later decisions, it is probably as near as one can get in a simple explanation. For example, a method of pruning clove trees to stop the spread of a fungus disease was held not to be a manner of manufacture[4]. Another example of a patent application which failed for the same reason is a method of fumigating a building by means of an insecticidal aerosol[5]. Last year a method of increasing the yield of wool by administering thyroxine to sheep was held not to be a manner of manufacture[6].

The second requirement for patentability, that the invention shall have "novelty", means that, at the date when the patent application, which includes a description of the invention, is filed at the Patent Office, it must not be known, nor must it have been used, in this country. A problem arising from this requirement of the law is the need to restrain research workers from publishing their results before the appropriate patent application has been filed. Prior publication is a complete bar to obtaining a valid patent—but it must be publication in this country, including publication in a foreign journal available in the country at the priority date. Availability at the Patent Office Library is the standard method of proving publication, but of course any other form of publication is sufficient to destroy validity. And it must always be remembered that a communication does not cease to be a publication merely because it is marked "confidential". Disclosure to a colleague who is bound to treat his employer's affairs as confidential is however permissible.

So far as prior use is concerned, public working of the invention during the year preceding the priority date is permissible for the purpose of reasonable trial if the invention is of a kind which can only be tried in public. Presumably this would cover clinical trials of a new drug, although usually the pharmacological testing stage provides sufficient evidence to enable a decision to be made as to whether the filing of a patent application is warranted. It is not usual to shelter under this limited permission of public working because it relates only to British patents—and if it resulted in publication of the invention in another country before the British patent application was filed, a valid patent would not be obtainable in that country.

Apart from this special provision for public working prior use is a ground on which a patent application can be opposed by an interested party[7] or a patent revoked by the Court after it has been granted[8].

In most countries the Patent Office conducts a search of the literature to see if the invention has been described previously, but the thoroughness of this search for "anticipation" varies considerably in different countries. The British search is less extensive than some and a curious provision of our law[9] is that a disclosure in a patent specification whether British or foreign which is more than 50 years old does not destroy novelty although it might serve as supporting evidence of prior use.

The U.S. Patent Office is experimenting with electronic means of recording and searching the literature. At present this "Mechanised Division" is concerned only with steroids, and it seems to be handling what must be a difficult field with speed and efficiency. From the applicant's point of view it has the advantage that, so far at least, the machine is unable to think, and thus office objections based on taking ideas from several sources, combining them together and concluding therefrom that "no invention is seen in the application" are virtually eliminated—and such objections, based on opinion as to obviousness, are often extremely difficult to content. The British Comptroller-General of Patents has expressed the view that the means of searching that are available to examiners in the Patent Office are so highly developed that any mechanical or electronic alternative "will have to present considerable advantages in speed and cost to make its adoption worth-while[10]". I refer later to the possibility in our industry that serious loss may result when the grant of a patent is delayed and I suspect that many firms would prefer the speediest procedure even if the cost were somewhat higher.

Before leaving this subject, it should be mentioned that disclosure by word of mouth is just as fatal as disclosure in writing—but I hope this warning will not prevent research workers from enjoying all the amenities provided by those who entertain the British Pharmaceutical Conference.

The third requirement for patentability is that there shall be subject matter, or an "inventive step". The invention must not be obvious. The degree of inventiveness required to support a patent is very small; a mere "scintilla" of invention is sufficient[11]. The question as to whether an alleged invention is obvious is often one of great difficulty, for many admirable inventions seem obvious when once they have been made. Commercial success and supplying a long-felt want are among the criteria to be taken into consideration. Although just as essential for validity as any other of the requirements this question of subject matter does not present a serious hurdle to obtaining a patent in this country. The reason for this is that the British Patent Office does not concern itself with subject matter and the grant of a patent can be opposed by an interested party only if the invention "clearly does not involve any inventive step[12]". Yet when the patent is granted one of the grounds on which a Court can revoke it is that the invention "is obvious and does not involve any inventive step . . ."[13]; the word "clearly" is not used, and its inclusion in the earlier section relating to opposition proceedings gives the applicant the benefit of the doubt so far as consideration of his application by the Patent Office is concerned. Certainly the inclusion of so indefinite a qualification and the difficulty of proving a negative must make a potential objector pause before opposing on this particular ground. The present wording represents a compromise between the views of those who hold that the Comptroller, as an executive officer, should not have power of a judicial character on his own initiative to refuse applications on the ground of lack of subject matter, and of those who think it wrong that a patent should be granted for an alleged invention which obviously lacks inventive merit[14].

Chemical Inventions

This question of an inventive step acquires a different significance when applied to chemical inventions such as those with which the pharmaceutical industry is concerned for it is rare for a new chemical process to be involved. Most chemical process patents are concerned with the manufacture of a new compound or group of compounds by procedures well known in themselves—for example a Grignard reaction or the oxidation of an alcohol to an aldehyde—although not hitherto applied to the manufacture of the particular new compounds. In such cases it might be argued that there is no invention in applying procedures which would be obvious to any chemist desiring to make the new compound. The position was explained in the well-known "sulphathiazole case"[15] where it was pointed out that an invention consisting of the production of a new substance from known materials by known methods is not patentable merely because the product is new, but it may be held to possess subject matter provided the substances are truly new (not being all merely additional members of a known series) and useful, and their useful qualities are the inventor's own discovery. In other words, in chemical process cases the real inventive step is often the discovery of the value of the products.

It follows that in chemical patents the question of subject matter is often inextricably bound up with the question of "utility"—the fourth requirement for patentability. An invention can be the subject of a valid patent only if it is useful and two aspects are involved. The procedure described must be useful in the sense of producing the result stated by the inventor and that result itself must be useful, in the sense of giving some advantage to the public. Lack of utility is one of the grounds on which the Court can revoke a British Patent[16], but provided the process "works" a very small degree of usefulness in the result is sufficient. In other countries, especially the United States, Germany, and recently Denmark, this question of utility is of the utmost practical importance and it is dealt with in detail later.

Scope of Provisional Specification

If then a patentable invention has been made, a description of it has to be filed at the Patent Office. This description is known as the "specification" and in the U.K. and other, mainly Commonwealth, countries which follow British practice the specification usually filed with the application when it is first filed is known as a "provisional" specification. The only legal requirements about a provisional specification are that it shall describe the invention and begin with a title indicating the subject[17]. At a later date a complete specification must be filed. It must "particularly describe" the invention and the best method of performing it known to the applicant; it ends with claims defining the scope of the invention claimed[18]. The complete specification is required to be filed within a year of the filing of the provisional although by paying additional fees an extension of up to a further three months can be obtained. The general theory behind this two-specification practice is

that in the provisional the inventor discloses the general features of his invention and early experimental results, and then he has a period of several months to work on it so that in the complete he can disclose the full details and indicate the exact scope of the monopoly claimed. His priority runs from the date of filing the provisional so far as he describes the invention in it. Before 1950, when the Patents Act, 1949, came into operation, the invention could undergo "legitimate development" between the filing of the provisional and the complete specifications without losing the priority date of the provisional. Under the present Act, a claim which is "fairly based" on the provisional specification has priority from the date of filing the provisional; otherwise the priority date is that of filing the complete which, as indicated above, is usually a year later. Although the application to particular cases in the pharmaceutical industry of the principle of "legitimate development" was fraught with uncertainty, the change in the law has not, in my experience, reduced it from a commercial point of view. If, for example, in a reaction for which a patent was sought before 1950, an alkyl group was introduced and the provisional specification disclosed that valuable compounds were obtained when the alkyl group contained up to, say, 8 carbon atoms, one would feel that disclosure in the complete that a valuable compound was obtained with an alkyl group containing 10 carbon atoms was "legitimate development", and therefore a claim covering this compound would have priority from the date of filing the provisional. But what would a Court have decided about a compound with a C_{16} alkyl group? Under the present law it has been held[88] that in deciding whether a complete specification is "fairly based" on a provisional answers to three questions are required. The first one is: Is the alleged invention as claimed broadly described in the provisional? If the answer is in the affirmative the next question is: Is anything included in the invention claimed inconsistent with the provisional? If there is no such inconsistency, the third question is: Does the claim include as a characteristic of the invention a feature as to which the provisional is wholly silent? Although this guidance is undoubtedly helpful, its application in particular chemical cases often leaves a feeling of uncertainty. Perhaps it is the uncertainty which is the main worry, for in this kind of case neither the patentee nor his competitor can be confident whether a compound is patented from the date of the provisional or from a year later. In a rapidly developing field—and in our industry most fields of research can be so described—a difference of one year in priority can be of the greatest importance.

In some countries, for example Holland and Australia, unlike this country, the priority date is inserted after each claim and in just such a case as I have mentioned the Australian Patent Office has refused to give the earlier priority to a claim to a compound which had not been specifically mentioned in the provisional although clearly within its scope. This does not necessarily mean that it would not be possible to restrain a competitor who discovers the C_{10} compound between the date of the provisional and the date of the complete. He will be barred by a valid generic claim to alkyl compounds as a group, but if for any reason the

generic claim is held to be invalid, perhaps because a research chemist once made a compound in the group, one cannot fall back on the more specific subsidiary claim.

Difficulty in deciding the scope of a provisional does not only arise from uncertainty on chemical grounds. I have pointed out that the inventive step in most medico-chemical inventions is the discovery of the value of the products. If, at the time the provisional is filed in my hypothetical case, the value of alkyl products as a group has not been ascertained it does not seem to me that priority for the alkyl compounds as a group can fairly be claimed.

I understand that those with wide experience of patents in various fields do not accept the view that chemical cases present greater difficulties than others in regard to the scope of the provisional. Be that as it may, the problem in practice is often one of the greatest difficulty. The research team make a few compounds of a group and biological investigation shows that each of the compounds has significant activity of the same type. It is obviously desirable to file a patent application immediately. But what should be covered in the specification—all the compounds in the group, only those actually shown to have activity, or some intermediate selection such as those which seem fairly easy or cheap to manufacture? And of course I have simplified the practical problem by referring to compounds of a "group". Bearing in mind the infinite variety of substitution that is possible in organic chemistry it is impossible to define a group in terms which will include all the compounds reasonably likely to be active and exclude the rest even if "reasonable likelihood" of showing activity was sufficient justification for a patent. It was of course just this dilemma which created the circumstances that led to the "sulphathiazole case" although the point on which it was fought was different. The problem was referred to by Lord Justice Somervell in the Court of Appeal[19] who said that he thought he appreciated the point that difficulty arose for chemical inventors if they could claim only one or more specific tested compounds when the probability is that other similar compounds have the same qualities but then he went on to say: "The conclusion I have come to . . . does not I think preclude the possibility of a chemical inventor obtaining adequate protection under existing patent law if he will take the proper steps". I do not know what steps the learned Lord Justice had in mind.

A legal authority with much experience in these matters recently suggested to me that the provisional should include a statement expressing the reasonable expectation of the extent to which the invention will apply to compounds other than those specifically mentioned. A claim to such compounds would then probably be regarded as "fairly based" on the provisional and the inventor would therefore have the additional year to find out how far the expectation was justified and to draft his claims accordingly. This suggestion may go a long way to solving the problem in drafting many chemical patent specifications in the U.K. When, however, the invention resides in the discovery of therapeutic activity, the unpredictability of the relation between the degree of activity and,

say, the length of side chain may often make it extremely difficult to formulate the expectation. Further, insofar as foreign applications are concerned, I suspect that in many countries there would be difficulty in securing acceptance of the "reasonable expectations" as being within the scope of the invention.

The only safe procedure seems to be to make and test as wide a range of compounds as possible before filing the application so that it can be based on ascertained facts, or else to file a succession of applications as the biological results are obtained. Commercially, the delay entailed by either procedure may mean that someone else gets in first and you lose your patent and much of the financial benefit of your research. It seems to emerge clearly from this that the larger the research team, and the greater the speed with which the ramifications of a chemical invention can be followed up, the greater the prospect of obtaining broad and valid patent cover. But anyone familiar with the problems of getting clinical trials carried out on the products of industrial research will know that a year in which to determine the scope of a patent, so as to be able to claim all—and only—the useful compounds, is often inadequate. A patent agent with considerable experience in the pharmaceutical field has said[20] that it is almost impossible to pay more than lip service to the legal principles for validity and pointed out that the effect is to encourage what he called "dishonest" statements although I should prefer to describe them as "optimistic without much foundation". He expressed the hope that in any case where validity is challenged because some of the compounds claimed do not have the virtues alleged, the Courts will take a generous view of the difficulties—and we must leave it at that.

Convention Patents

In industrial practice this problem does not end with consideration of the scope of a provisional in comparison with what it is intended to include in the complete specification in the United Kingdom. It affects the position overseas. As many of you will know, under the International Convention for the Protection of Industrial Property, first arranged in 1883 and subsequently amended, a British inventor who has filed an application and a provisional specification in the U.K. can apply for a corresponding patent in any country which has adopted the Convention —known as a "convention country"—within one year of his U.K. application, and still obtain priority as from his U.K. filing date. Similarly an inventor who files a patent application in an overseas convention country can file a corresponding application in the U.K. within one year of his original date of filing, and obtain priority as from that original date. Advantages of this arrangement for the British inventor are that the British complete specification and the corresponding foreign specifications can be prepared at the same time, and the inventor has a year after making his invention within which to decide whether its commercial value is such as to make it worth while to incur the quite substantial expense of applying overseas. The invention claimed overseas must be the same as that disclosed in the British provisional, and at

once the old problem arises—what is the scope of the invention described in the provisional? Practice varies in different countries—some Patent Offices take a liberal view as to the scope of the invention described in the provisional, others take a narrow view; and inevitably, as the human element is concerned, the same Patent Office does not always seem to be consistent.

Although applications filed overseas in accordance with the Convention can claim priority as of the date on which the corresponding application was filed in the country of origin, in all other respects such applications are subject to the same law and practice as apply to an original application in the country concerned, and these often differ from British law and practice. I should like, therefore, now to refer to a few of the outstanding examples of such differences.

"*Interferences*"

First, I will mention the law in the United States on priority. Whereas in most countries the priority date is that on which the application is filed, the date in U.S.A. is that on which the invention was discovered or thought of by the inventor— the "date of conception". The inventor can even publish his invention but still get a valid patent if his application is filed within a year of such publication[21], although he will not be able to obtain a valid patent in any country outside U.S.A. where the publication becomes available before the U.S. filing date. The determination of priority by reference to date of conception obviously creates a difficulty when two or more inventors file applications covering the same ground, for the Patent Office examiner cannot know which inventor had the earliest date of conception. In such instances the procedure by which the applicant with the earlier date of conception is ascertained is known as an "interference", the inquiry being conducted by three "examiners of interferences"[22]. I have sometimes come across research workers in this country who have had an impression that all that is necessary for a U.S. worker to show the date of conception of his invention is to produce the appropriate laboratory notebook—and there may even be the faintest suggestion that the date appearing in the notebook is not above suspicion. Any such impression is quite without foundation, and Americans themselves have the greatest respect for the thoroughness of the procedure by which priority is determined. The effective date is not merely that on which the idea occurred to the inventor—the invention is not complete until it has been "reduced to practice"—a physical act as distinct from the mental act. In chemical cases this means that it must have been shown that the reaction took place as described. The inventor's own and uncorroborated evidence is useless; corroboration must be by someone other than a co-inventor who understands what is being written and can testify from his own knowledge that the work described in the notebook was actually done[23].

In an interference the first stage is that each party submits a sworn preliminary statement specifying the dates when the acts relied upon as

showing he had completed his invention were done, and the party will not be allowed to claim earlier dates in the subsequent procedure. After the statements have been accepted by the Patent Office each party can see the other's patent application but not his preliminary statement so that neither knows as yet the other's claimed priority date. The next stage is the collection of sworn evidence, which is given in the form of question and answer each witness being questioned by the attorney for his own side and cross-examined by the attorney for the other side. Finally, there is a hearing before the Board of Interference Examiners. I understand that in practice the result of most decided interferences is that the party who files first obtains priority and one wonders whether such an expensive and lengthy procedure is really worth while in comparison with the law in our own country and most others which gives priority to the first applicant. I have earlier mentioned that in a keenly competitive industry such as ours any delay in obtaining a patent may lead to other manufacturers marketing the same product and one would expect that the considerable delay created by the interference procedure would greatly facilitate this kind of competition. It is therefore not surprising that a large proportion of interferences, especially it is understood in the pharmaceutical industry, are settled by negotiation between the parties. Before leaving this subject I must point out one anomaly from the point of view of the non-American inventor. If the interference declared is between an original application in the United States by, say, an American inventor and an application under the International Convention by, say, a British inventor, the latter cannot claim his "date of conception" —his priority date is that of the original U.K. application, but the American can go back to his date of conception. Furthermore, as the British priority date can be ascertained from Patent Office records, the American inventor knows the latest date he can claim if he is to win the interference. He can use this information to claim for himself a later date than he otherwise would have done and thereby be able to produce more adequate evidence of reduction to practice before the British filing date. In Canada, where a modification of this interference procedure is in force, the convention applicant from overseas is not restricted to the convention date, and proof of "reduction to practice" is not required. The Board of Patent Interferences decides the issue on the basis of an affidavit filed by each applicant setting out the history of his application.

Utility

Another matter on which difficulties arise in prosecuting overseas applications is that of utility. I have mentioned that, in general, everything within the scope of a claim must be useful and in the case of a British patent the extent of the usefulness can be quite small. In some countries, this question of utility is of the utmost importance, especially in America, where, in connection with chemical patents in the pharmaceutical industry, it has been the subject of much controversy in recent years. A U.S. patent can be granted for a "new and useful process, machine, manufacture or composition of matter"[24] and the specification

is required[25] to describe "the manner and process of making and using" the invention.

It has been held in the U.S. Courts[26] that the specification must include an assertion of utility and an indication of the use intended. If the invention is a pharmaceutical chemical—does this mean that one must indicate in the specification the pharmaceutical use or is it sufficient to show that the invention is useful as an aid to further research—in the words of the U.S. Constitution[27] "to promote the progress of science"? And if a pharmaceutical use is required, is it sufficient to show pharmacological activity without tests on human beings? Until relatively recent times the U.S. Office, like the British, were content with a very slight showing of utility and in fact they adopted the concept that an invention had utility provided it was not inoperative. Then a few years ago the U.S. Office—or at least some examiners in it, for they were not consistent—tended to require proof of clinical value. Recently, in my experience, assertion of pharmacological activity of a kind which would lead one to expect useful action in man has been accepted, perhaps because of a case in 1957[28] in which the demonstration of antispasmodic activity in laboratory animals was held to be sufficient. It must however be said in defence of the Patent Office's former attitude that there are numerous cases in which the Court has indicated that the Office must satisfy themselves that an assertion of therapeutic value is justified—and whatever may be thought of such judicial opinions the executive officers can scarcely be criticised if they act upon them even though no comparable proof is required in non-medicinal fields.

Utility of Intermediates

The question of utility in connection with applications in U.S.A. arises in a particularly troublesome form in connection with patents for intermediates. Until after the war, a statement that a compound was useful in organic synthesis was sufficient. Then it became necessary to indicate the compound which it was proposed to make from the intermediate, and that compound had to be of known value or its value had to be shown in the specification. The objection to this from the manufacturer's point of view was that it necessitated making further progress in a research project—in fact making a second invention—before patent protection could be obtained for the intermediate. A year or two later the U.S. Patent Office stiffened the requirements arguing that the intermediate itself had to have some valuable property. This led manufacturers to engage in quite useless research work to find some activity in the intermediate which could be put forward for patent purposes however inadequate commercially. It was permissible to supply this kind of information when the examiner asked for it, usually quite a long time after the patent application had been filed, and by that time the final product had probably been made and tested so that one knew whether it was worth while spending time and money on obtaining adequate patent protection for the intermediate. Then a further stiffening occurred and for the last year or two the Patent Office has adopted the

attitude that the utility of the intermediate—the method of converting it into a useful end-product—must have been included in the original specification as filed and where this has not been done a patent has been refused. Quite recently a further step has occurred. Priority in accordance with the International Convention has been refused in a case where the British provisional did not include this U.S. type of utility statement.

These changes in U.S. Patent Office practice have stemmed from court decisions, although they have been much criticised by the industry and have caused a good deal of irritation. For example, in a case in 1954, the Examiner had rejected an application claiming a compound which was stated to be of value in preparing more complex phosphorus derivatives and as a constituent of parasiticidal compositions. On appeal the rejection was upheld on the ground that there was no disclosure of the nature or of the utility of the more complex derivatives which could be obtained from the new compound, even though the specification referred to two other applications in which the intermediate was used[29]. Although not germane to the present point, the second kind of utility disclosed—the use in parasiticidal compositions—was also held to be insufficient because the specification did not say what kind of organisms were killed and therefore the applicant did not give an adequate disclosure as to how to use his compound. The Board of Appeals even suggested that a parasiticidal composition ought to have been exemplified. As a result of many protests from the pharmaceutical industry the Commissioner of Patents in 1956 defended the Office attitude in an address to the Division of Medicinal Chemistry of the American Chemical Society. On the question of proving therapeutic efficiency he claimed that the Patent Office had always sought proof of efficacy where a compound was claimed to be of value in a disease known to be difficult to treat and cited a case in 1940[30] in which a patent was refused for a preparation alleged to promote the growth of hair. He also argued that as the public believed that the grant of a patent implied that the patented product had value for the indicated uses and that it had Governmental approval, it was a responsibility of the Patent Office to protect the public by an appropriately cautious attitude. The Commissioner's arguments in relation to intermediates and to therapeutic efficiency were subsequently refuted in a memorandum to the U.S. Patent Office by the Sub-committee on Utility Practice of the Committee on Chemical Practice of the U.S. Patent Law Association in which a large number of legal decisions were reviewed, but little change has so far resulted, perhaps because the criticisms in the memorandum applied more to the decisions than to the Patent Office which is guided by them. It is not appropriate for anyone in this country to comment on the U.S. Commissioner's conception of the duties imposed upon him by the laws and Courts of his own country. We can, however, be thankful that our own Patent Office does not find itself obliged to be a judge of clinical trials, and pharmaceutical manufacturers, whether in America or elsewhere, may fairly doubt whether the highly effective work of the U.S. Food and Drug Administration in

controlling the marketing of new compounds in that country really needs supplementing by the presumably less expert activities of the U.S. Patent Office.

The latest stage in the controversy occurred about a year ago when the Court of Customs and Patent Appeals gave a decision which if it is accepted as representing the law makes the position in regard to patents for intermediates much more satisfactory[31]. The case was concerned with claims for two derivatives of 14-hydroxy-Δ^5-androstene. The specification referred to the presence of a 14-hydroxy group in the cardiac glycosides and said that the compounds claimed in the patent were valuable intermediates in the preparation of analogous 14-hydroxy steroids into which they could be converted by hydrogenation. The application was rejected by the Patent Office on the ground that the specification failed to show how these intermediates could be converted into useful compounds. The rejection was upheld by the Board of Appeals but the C.C.P.A. by a majority allowed the application and said the Office had been confusing the need for "utility" with the separate legal requirement that the specification should indicate the manner of using the invention. They returned to the old concept that "utility" in patent law means simply "not frivolous, or mischievous or immoral".

A lighter touch in the judgment of the Court was a comparison of the action of the Board of Appeals in rejecting this application because of lack of utility although it provided "new building blocks of value to the researcher . . . which have utility as intermediates in the search for cheaper and shorter routes to the synthesis of useful steroids", with their action in allowing a patent for a lacquer for changing the contour of the human nose[32] on the ground that the improvement of the features of a person had utility. On the question of revealing how the invention is to be used, the Court said that the specification told those skilled in the art that steroids having analogous structures were made by hydrogenation, and that was enough. Research workers would know how to use the new compounds. To require that the specification should show how to make at least one therapeutically valuable compound from the compounds in the patent would be "quite an effective way to remove the stimulus of the patent system from this kind of research".

The Court went on to refer to the problems of the Patent Office in handling applications in the field of pharmaceuticals where assertions are made of beneficial therapeutic effects on human beings and said that it is entirely proper that such assertions be carefully investigated. Thus, the Patent Office's incursions into the field of therapeutic trials seem to be supported.

It is understood that this decision is to be the subject of an appeal, but as the law stands at the moment in U.S.A. as a result of this case a patent for an intermediate cannot be rejected for lack of utility merely because the compounds are not themselves of therapeutic value. It is however necessary in the specification to indicate how the new compounds may be used to give compounds which are, or may be, of value, though, it may be, merely as intermediates for yet other compounds. It is a

further effect of this case that the Patent Office are supported in requiring proof of clinical effectiveness in any case where the Examiner thinks there is a doubt.

Perhaps a foreign observer may be permitted to express the hope that a reasonable view will be taken of what constitutes such proof. In a recent case of mine, admittedly not a strong one, an examiner rejected an application on the ground that the evidence of more than one medical man was necessary, and that each doctor should have treated at least ten patients. While the latter may be somewhat arbitrary there is judicial support for a requirement of thorough testing and successful trial by at least two physicians[33]. On the other hand the Board of Appeals decided in favour of an applicant in a case in which a product for the treatment of duodenal ulcer was stated in a doctor's affidavit to relieve some of the symptoms. The Patent Office claimed that radiological evidence of cure should have been presented, not merely the subjective statements of patients, but the Board held that relief of symptoms such as flatulence and pain could only be shown by questioning the sufferers[34].

America is not the only country where the patenting of intermediates may run into difficulty. Germany is another example where in general such patents are refused and the Patent Office attitude has recently been vigorously defended by the Chairman of the Senate in the German Patent Office[35]. Patent applications in Germany are rejected if they relate to processes for making intermediates which processes are analogous to known processes. An "analogy process" is one in which the mode of operation is the same as in a known process and the problem to be solved is the same, so that a chemist could assume with a probability bordering on certainty that the conversion of the new starting materials will proceed in the known way and give a product of the kind expected. A patent is allowed for an analogy process by the German Patent Office only if the product is new and has a novel, beneficial and not obvious use. It is considered that an intermediate does not have this kind of use, even if it can be used industrially.

Earlier this year, the highest tribunal in patent matters in Denmark came to the same conclusion.

Examination of Applications

Let us now return to the U.K. and assume that the foregoing hurdles have been borne in mind and a complete specification filed. In spite of skilled professional help in drafting a specification it almost invariably happens that the patent office examiner will find some fault—be it an ambiguity, a lack of clarity or just a plain error—in the wording. Sometimes a claim will be criticised as being too broad having regard to what is stated in the body of the specification. Although by the nature of his job an examiner has to be a somewhat severe critic, it must be borne in mind that his comments help to reduce the risk that any patent eventually granted will be held to be invalid. Further, if sometimes a criticism is

difficult to deal with, a discussion with the examiner will often produce a mutally satisfactory answer and I must include examiners at the Patent Office among the most helpful of our often unfairly maligned civil servants. A provisional specification is not examined until the corresponding complete specification is being considered. It used to receive its own attention and a common criticism was that there was disconformity between the complete and the provisional. As indicated above such disconformity no longer matters in the U.K. since matter in the complete specification but not in the provisional is acceptable, but its priority runs only from the date of filing the complete. One of the commonest criticisms is of the title, the legal requirement being[36] that the title shall indicate "the subject to which the invention relates". A list of applicants and the titles of the applications is published weekly and in order to avoid disclosure of the direction of one's research, it is usual to give as vague a title as possible when filing the original application—such as "Improvements in or Relating to Organic Compounds"—and the Patent Office in due course requests something more specific but this will not be published until the application is accepted.

Eventually if all the objections are overcome, the applicant is notified that his application is accepted by the Office and that the specification will be published. Three months then elapse during which the grant of a patent can be opposed, but if no opposition is filed "letters patent" will in due course be received.

The length of time during which an application is being considered is of course dependent on the time taken to reply to objections raised by the Examiner. The specification is required to be in order for acceptance within 3½ years of filing the complete specification, subject to a maximum extension of three months on paying the appropriate fee[37]. Failure to comply entails rejection of the application. Damages for infringement can be obtained only from the date on which the specification is published[38] and the marketing of the product before that date may result in competition from imitators who are quick off the mark. The greater the usefulness of the product and the easier it is to make, the more likely it is that such competition will occur. Subject to the possibility of obtaining a licence under the patent, a matter which is dealt with later, legal action can be taken against the competitor to force him to withdraw the imitation when the patent is granted but in the meantime the patentee may suffer considerable damage for which he has no redress. In America it is possible to secure early consideration of an application in certain circumstances, such as where an infringement is occurring or the absence of a patent hinders commercial negotiations for exploitation of the invention. This seems to be a precedent which might with advantage be adopted in this country.

As a matter of interest I may mention that the average time for a representative half dozen patents with which I have recently been concerned to go through the Patent Office, from the date of filing the provisional specification to the date on which the patent was granted, is nearly 2½ years, and any inventor here who is thinking of filing a patent

application may like to know that the average cost—in the United Kingdom only—was nearly £80.

Employees' Inventions

A matter that may be mentioned at this point is the ownership of patents as between employer and employee. It is of course possible for the question to be dealt with in a written agreement or contract of employment in any way the parties may decide, but in the absence of specific agreement the general rule is that "where the employee in the course of his employment (that is, in his employer's time and with his materials) makes an invention which it falls within his duty to make . . . he holds his interest in the invention, and in any resulting patent, as trustee for the employer"[39]. This rule covers most of the cases in the pharmaceutical industry since obviously it *does* fall within the duty of research workers to make inventions. But sometimes the position is not quite so simple. Suppose for example a senior employee such as a manager in change of a packing department invents, say, a machine for counting tablets. In such cases the law regards it as inconsistent with good faith that the employee should own the invention. As a senior employee he is expected to use all his abilities in the service of the employer. But if the same kind of invention is patented by a relatively junior employee, the patent would belong to the employee and the employer would have no right to it even though the employee used some of the employer's materials and time. In America in such cases the employer has what is known as the "shop right" to use the invention in his own business without payment, while the employee can exploit it for his own benefit outside the employer's business, but in British law the patent usually belongs wholly to the employer, or, rarely, wholly to the employee. Prior to 1949, disputes as to ownership between employer and employee could be settled only by an action in the High Court, a procedure which would naturally be beyond the financial resources of the employee. Under the current Act these disputes can be heard by the Comptroller, with a right of appeal, although the Comptroller can decline to deal with the matter if he thinks it could more properly be dealt with by the Court[40]. Further, the Comptroller has power to apportion the benefit, but only if he is not satisfied that one or other of the parties is "entitled" to the benefit to the exclusion of the other[41]. In the Patchett case[39], the word "entitled" was held to mean "having a legal right", and since the law, whether fairly or not, has almost always given the sole right to one of the parties, usually the employer, the power of apportionment is likely to be exercised only very rarely—perhaps only when there is a written contract of employment which provides for sharing the benefits of the employee's inventions without specifying the respective shares.

It may be that employees not engaged on research who make inventions have some justification for regarding the law on this matter as being unfair to them. Certainly before the Patchett case it was widely thought that under the 1949 Act disputes of this kind between employer and

employee would often be settled on the basis of sharing the benefits and this view of the intention of those responsible for that Act is supported by the Final Report of the Swan Committee (see especially para. 27). There does not seem to be the same objection to the vesting in the employer of the exclusive right to an invention made in the course of his employment by an employee engaged in research. Even if an employer in the pharmaceutical industry wishes to give some reward to the employee who "invents" a new and valuable compound, it is difficult to do so fairly. There is no certainty that a new compound will have the therapeutic value it is hoped it will have and major factors in the success of research work, however well-informed the concept, are volume of work and just plain luck. Success in research, in the commercial sense, cannot therefore be regarded as an indicator of special merit, for the worker who makes the compounds that turn out not to have the hoped for properties may well be as brilliant a chemist and have made as significant a contribution to the total research effort as his more fortunate colleague.

Who is the Inventor?

While on the subject of employees I should like to mention a problem to which there seems to be no simple answer. In applying for a patent the name of the "true and first inventor" must be disclosed. If untrue information is given the Crown is deceived and the patent is invalid[42]. I am told that inspiration comes to a chemist at quite unlikely times and places. He discusses the idea with his colleagues over the morning coffee; a plan of work is formulated and the various jobs given to members of the team. In the course of the research some ideas are found to work, others have to be changed. Eventually a compound emerges which is tested by the pharmacologist and found to have a useful action, following which it is tried out clinically and shown to have a valuable therapeutic effect. Who is the true and first inventor? Is it the chemist who originally thought the compound would be worth making and testing? And are those who followed to be regarded as the mechanical means of carrying out and verifying his ideas? I have already quoted high authority for the proposition that the process of making a new chemical compound by known procedures may be patentable if the new compound has useful properties which are the inventor's own discovery. It may be the pharmacologist who discovers the useful properties although it is the chemist who devises the manufacturing procedure that is patented. It is submitted that in such cases the chemist and pharmacologist are joint inventors.

Again, some organisations carry out as a routine a wide range of pharmacological tests on every compound made, including compounds made for completely non-medical purposes. Suppose in the course of such routine work a compound unexpectedly turns out to be of value for, say, treating cancer. Assuming that as a result of that discovery the method of manufacture constitutes a patentable invention, it seems that the pharmacologist would be the inventor.

The application can be made by the true and first inventor or by his assignee, and in each case either alone or with any other person In industrial practice the application is often made by the company as assignee of its employee, but the official application form requires a declaration that the inventor assents to the application being made. When a complete specification is filed a further declaration of inventorship must be made whether or not the specification contains new matter invented by someone other than the original inventor[43]. When making these declarations it is common practice to include among the applicants those who have made the main contributions to the conduct of the chemical research and to regard this limited group as being a collective first and true inventor. But it does not seem certain that a Court would take a benevolent view of this practice. It can be argued that making an invention, being a mental act, cannot be made by a team. It can also be argued that the group should include all who have contributed ideas that have been utilised in the course of the work. The point has not been tested in the courts but if there is a vital legal significance about naming the inventor correctly it seems desirable that the law should be modified to accord with modern research practice and to eliminate a somewhat technical trap which confers no particular benefit on anyone.

Before 1949, an application could not be prosecuted by an assignee so that the employer was not free to deal with the invention before the patent was granted. The employer could take an assignment of the application so that the patent was issued to him, but his rights were not recognised before the issue took place. Difficulty therefore occurred during the application stage if an employee applicant changed his employment or went abroad and the employer desired to grant a licence or otherwise deal with the invention. This has now been altered and an employer can claim sole ownership from the date of the application while the inventors can have their names shown on the specification when it is published and thus secure the personal credit attaching to the invention—a simple change in the law that has saved a great deal of unnecessary trouble.

Product Patents

From the chemical and pharmaceutical point of view the outstanding change made by the 1949 Act was the introduction—or, rather, reintroduction—of product patents, as a result of which patents became obtainable for two kinds of product, previously unpatentable.

Perhaps the most important prohibition removed was that on the right to obtain a patent for a new chemical compound as such, as distinct from a compound when made by a particular patented process. Before 1919 it was the practice for patent specifications to include claims to chemical substances at large. The compound, however made, was then the monopoly of the patentee. The British chemical industry at that time was very much in its infancy and, especially in the section concerned with the manufacture of dyes, was struggling against the post-war

rejuvenescence of German competition. The industry in general welcomed the statutory prohibition contained in the Patents and Designs Act, 1919, of "claims for the substance itself, except when prepared or produced by the special methods . . . claimed or by their obvious chemical equivalents"[44]. Some difficulty subsequently arose over the interpretation of the word "special"[45] and the wording was changed in 1932 to "except when prepared . . . by the method or processes of manufacture particularly described. . .".

Under this provision a firm which discovered a valuable compound made by a chemical process and patented a method of making it might find itself in competition with another firm which had devised an alternative method of manufacture. One result was that the discoverer of a new compound endeavoured to patent all the possible ways of making the compound that he could think of, thereby wasting much effort and obtaining patent protection for a number of processes which would not be used. Further, legislation of this type must tend to deprive the discoverer of a new and valuable compound of the fruits of his invention. Indeed, as I have indicated, the object of the provision was to enable British chemical manufacturers to make useful compounds, especially dyes, discovered by foreign competitors provided they could devise a non-infringing method of manufacture.

By 1946, the attitude of the chemical industry had changed and we find the Joint Chemical Committee on Patents, a body representing a number of industrial and scientific organisations concerned in the practice of chemistry, including the Association of British Chemical Manufacturers and the Wholesale Drug Trade Association, recommending the restoration of the power to patent substances[46]. The Committee pointed out that the inventive step is often the conception of the compound and that the method of making it may well be obvious to a chemist. The recommendation was accepted by the Swan Committee[47], and the 1949 Act made the appropriate alteration in the law. In America and Canada product patents have always been obtainable, and some Commonwealth countries have followed the current British Act, but most countries in Europe still limit the scope of their patent protection to chemical processes. The desirability of product patents for chemical substances is a matter for endless argument. If it be considered that patents in the chemical field are beneficial as facilitating and stimulating research then the better protection afforded by a patent for a substance however made as compared with a patent for the substance when made by a specified method must be regarded as desirable. If on the other hand you regard a patent as conferring a monopoly which must be conceded reluctantly and to the minimum practicable extent then you may think that the removal of a limitation on the scope of patent protection is unfortunate. And it can of course be argued that there is a definite stimulus to research if it is open to others to exploit alternative methods of manufacture, while the reply to this will be that a greater good is served by devoting the research effort to the discovery of new valuable compounds than to the evasion of a competitor's patent.

Patents for Food or Medicine

On whichever side the balance of advantage to the public may be thought to lie, the history suggests that an industry which is confident of its future and actively carrying out research prefers the product patent.

The second restriction on the grant of product patents which was removed by the 1949 Act was that relating to substances intended for food or medicine. In patents for new chemical compounds of therapeutic value it is now a common practice also to include a claim to pharmaceutical compositions incorporating the new compound which, it is submitted, would not have been allowable before 1949 as being substances intended for medicine. The following is an example[48]:

> "A therapeutic composition having prolonged adrenal cortical hormone-like activity comprising a sterile solution of 17-hydroxy-corticosterone 21-beta-*cyclo*pentylpropionate in a non-toxic fluid vehicle".

A further advantage of this change in the law is that it is now possible to obtain here, as in U.S.A., a patent for a therapeutic composition based on the discovery that a known chemical compound has a valuable therapeutic action which was previously unsuspected. The "composition" may be made by some well-known procedure, such as tabletting or mere mixing, which cannot be patented because it is not new; but if the resulting composition has not hitherto been made, and if it is useful because of the newly discovered therapeutic value of the active ingredient, then a product patent is obtainable for the composition.

Patents are however still refused if the invention claimed is a mere mixture of known ingredients possessing only the aggregate of the known properties of the ingredients[49] and a claim to a new substance does not extend to the substance when found in nature[50].

Use of Patent Monopoly

Let us now turn to the ways in which a patentee can make use of his patent. The wording of letters patent indicates that the grant confers on the patentee the sole right to "make, use, exercise and vend" the invention. *The Times*[51] has suggested that a patent is "sometimes cynically described as a passport to litigation in the courts", for the inventor must himself enforce his right, and the practical effect of the grant is only to entitle him to take legal action to restrain anyone who is making, using, exercising or selling his invention without permission. In recent months I have come across more than one pharmacist who is under the impression that if a substance or preparation which is the subject of a patent is included in the B.P. or B.P.C., the patent can be ignored. This impression is completely without foundation and I may remind you of the "Notice Concerning Patents" in the B.P.[52] which states that the inclusion of such products "neither conveys, nor implies, licence to manufacture".

The right conferred by a British patent continues for a period of 16 years from the date of filing the complete specification provided that

the requisite annual renewal fees are paid. A period of four years from this filing date is allowed before any renewal fees are due and thus the patentee has a little time within which to decide whether it is worthwhile maintaining the patent. The renewal fee payable before the end of the fourth year is £5, and thereafter it increases each year up to £20 for the final year. In America there are no renewal fees and the patent continues in force for 17 years. Canada, as in many other patent matters, follows the American practice, but most countries require that renewal fees be paid if the patent is to remain in force.

In the United Kingdom only half the normal renewal fee is payable if the patent is at the request of the patentee endorsed "licence of right", which means that anyone can obtain a licence to use the patent on terms which, if not agreed between the patentee and the licensee, will be fixed by the Comptroller[53]. Little use seems to be made of this provision in the pharmaceutical industry although one might think that in view of the special position of medicines in regard to licensing, which I shall mention in a moment, the facility is especially suitable for patents in the medical field.

The patentee who brings an infringement action must prove infringement and where a process is involved proof is often difficult, since it is necessary to show what is being done in the alleged infringer's factory. In the case of an imported substance proof of infringement of a process patent is virtually impossible—another reason why product patents give more adequate protection. In America the Customs authorities have power to prevent the importation of a product which if made in America would constitute infringement of a U.S. patent[54].

When it does occur, infringement is likely to be accidental. I remember being asked by a colleague to arrange for a patent application in respect of an improvement in a certain process and the details seemed vaguely familiar. To his utter astonishment I eventually turned up in my files a copy of a specification describing the improvement which had been signed and dated by him, showing that he had read it some two years earlier. He had long since forgotten the patent and then some quirk of memory had recalled the idea which he had developed, incidentally on slightly different lines from those of the original inventor, but still based on the patented principle so that the improvement in question had to be discontinued.

It has been pointed out by the Patents Manager of the National Research Development Corporation[55] that the modern tendency in industry is to file patent applications to cover individually every little improvement in a process without giving overmuch thought to the question of validity. "The complexity involved in challenging such a 'web' would daunt a would-be trespasser". The author goes on to say that this tendency "is commonly expressed by saying that firms tend to respect each other's patents". I should like to think that in industry in general such respect rests on something more creditable than the complexity of challenging another's patents. In our industry, since the re-introduction of product patents 10 years ago, infringement of a patent

for a chemical compound is of course immediately obvious unless the compound is an intermediate which is not offered for sale. Infringement is therefore extremely unlikely, but even when patents were granted only for processes, and compounds when made by the patented processes, infringement actions were rare.

The usual answer to an allegation of infringement is that the patent is invalid—that for one or more of a dozen reasons specified in the Act[56], not all of which were considered by the Patent Office when dealing with the application, the Court ought to revoke it. The mere fact that the patent was granted has no bearing whatever on the question of validity. Meinhardt[57] states that during the period 1919 to 1949 the patentee was unsuccessful in 72 per cent of the infringement actions reported in *Reports of Patent Cases*, and in most instances the patent was held to be invalid.

Licences

If a patentee does not wish to keep for himself the exclusive right to exploit his invention he can either assign it to someone else or license one or more other people to exploit it on whatever terms as to remuneration and other matters are mutually agreed. He can, for example, specify the price at which the licensee may sell the product, and a licence agreement is not required to be registered under the Restrictive Trade Practices Act, 1956, if the restrictions which would otherwise necessitate registration relate only to the invention or articles made by the invention[58]. One kind of condition is not permitted in a patent licence, namely what is called a "tying clause", that is, a requirement that the licensee shall purchase other goods, not covered by the patent, from a specified person, or that he shall not use any articles or process except such as belong to the patentee[59]. Any such condition is void, but further it is made a defence to an action for infringement to show that a contract including such a void condition is in force at the time of the infringement, even with a third party. Of course the sting of this provision is somewhat lessened by the difficulty of proving the existence of a contract of that kind, but the possibility of the clause being invoked probably reduces the likelihood that a patentee will try to enter into such an agreement. If, however, after the action, the objectionable condition is eliminated from the licence the patent again becomes fully effective and the infringement cannot continue but of course the patentee may not be free under his agreement with the licensee to eliminate the condition. The sanction would, however, be more effective if the infringer, having successfully defended the action on the ground that an improper provision was included in a licence, was himself entitled to a licence for the remainder of the life of the patent.

As the patentee has the sole right to use and sell his invention, those who purchase the patented product from him, and any subsequent possessors, are deemed to have acquired a licence, so that they are able to use and sell it as they please. The patentee can attach conditions to the sale, and these conditions are binding on subsequent purchasers if

they acquire the patented article with knowledge of the conditions. The subsequent purchasers are said to have a "limited licence" and those of you who are in retail practice will no doubt recall having seen notices on patented articles on your shelves indicating that they are sold under a limited licence and subject to specified conditions. The commonest of such conditions is that the article shall not be sold below a specified price. If you sell such an article in breach of the condition you are no longer protected by your limited licence, and it is not generally realised that such sale is an infringement of the patent rendering the seller liable to an infringement action. An interesting example of this was the infringement of a patent for a tube by the sale of a brand of tooth paste in the patented tube at less than the price fixed by the patentee[60].

Contributory Infringement

In this country it is not an infringement to sell an unpatented article knowing it is going to be used for a purpose which is the subject of a patent. Suppose, for example, there is a patent covering an agricultural spray based on substance X, X itself not being the subject of a patent. No infringement action can be brought against anyone selling X with instructions for using it to make the spray. The farmer who uses it as instructed is infringing the patent, but it is obviously impracticable for the patentee to enforce his rights against a large number of individuals. A hospital pharmacist who manufactures a patented tablet may unwittingly be sheltering under the same umbrella. In America the person who sells a product, knowing that it is specially made or adapted for use for a patented purpose, is liable as what is called a "contributory infringer[61]". The doctrine of contributory infringement may provide valuable protection when a new use is discovered for a known substance and at least from the patentee's point of view might with advantage be incorporated in British patent law.

Cost of Litigation

Complaints are often made of the cost of patent litigation, usually on the ground that it prevents a relatively poor individual inventor from enforcing his rights against a large company whom he believes to be infringing his patent. It seems to me that however unfortunate this situation may be it is inevitable. Obviously any company alleged to be infringing will utilise the best technical and legal advice it can obtain—and such advice is necessarily expensive. The same is true if it is a company that makes the allegation. If the patent is commercially important the cost of litigation, however large by private standards, may well be insignificant in comparison with the financial effect of the decision. Some small changes in procedure were introduced by the Patents Act, 1949, with a view to reducing cost, but little can be done by legislation, unless it be the nationalisation of the patent agents, the legal and other professions. One rather curious alleged protection for the "small man" is that it is actionable to threaten anyone with proceedings for infringement although it is permissible to draw attention to the existence of a

patent[62]. I find it difficult to believe that a threat of proceedings is more intimidating than a mere reference to a patent which the recipient knows to mean the same thing, and it seems to me that this provision provides more of a trap for the unwary patentee than protection for the small inventor.

Abuse of Monopoly

In general the word "monopoly" arouses an emotional antagonism in the mind of the average member of the public and this is just as true of the monopoly conferred by a patent as of any other kind of monopoly. The law attempts to give some protection against abuse of a patent monopoly. This protection stems from the Statute of Monopolies which, as we have seen, did not extend permission for the grant of monopolies to those that were "mischievous to the State, by raising prices of commodities at home, or hurt of trade or generally inconvenient". The protection consists in giving the Comptroller authority[63] to grant compulsory licences or to mark patents "Licence of Right" if he is satisfied that abuse occurs. In accordance with an international agreement the patentee is given 3 years after his patent is granted before anyone can apply to the Comptroller. The kinds of abuse which can form the basis of an application are specified in the Act[64]. As the guiding principle in exercising these powers the Act states[65] that the object is to secure that the inventions which in the public interest should be worked in the U.K. are in fact worked without delay and to the fullest practicable extent. If the demand for the patented article is not being met or is being met only by importing it, if another invention cannot be worked without a licence under the patent and a licence on reasonable terms is refused, or if a condition attached to the grant of a licence prejudices some other industrial activity, an application for a licence can be made to the Comptroller.

At the same time the Act specifies that the patentee shall receive reasonable reumuneration having regard to the nature of the invention, while the ability of the proposed licensee to work the invention and the risks he will undertake in providing capital are also to be taken into account. The law thus imposes on the Comptroller the responsibility of acting as a financial and technical expert as well as a patent and legal authority, although evidence on these matters would of course be given.

The right to seek redress if a patent is used in an unfairly restrictive manner contrary to the public interest has been very rarely used. The Swan Committee[66] said that popular attention in regard to abuse of patent rights had been concentrated mainly on the deliberate suppression of inventions. The kind of allegation they had in mind was that a manufacturer might buy a patent with the deliberate intention not to use the patented invention because it would render obsolete some profitable activity he was carrying on. The Committee reported that several persons who had made public statements about this alleged practice did not accept an invitation to give evidence, and they suggested that the allegations might be explained on the basis of "the unfulfilled expectations

of an over-sanguine inventor" or on a failure to appreciate some of the commercial problems involved.

Compulsory Licences for Medicines

So far as patents relating to medicinal substances are concerned the provisions for preventing abuse of monopoly are never likely to be invoked while the law continues to provide, as it has done for 40 years, for the grant of compulsory licences under such patents. This provision deserves to be quoted in full[67]:

(1) Without prejudice to the foregoing provisions of this Act, where a patent is in force in respect of—

(*a*) a substance capable of being used as food or medicine or in the production of food or medicine; or
(*b*) a process for producing such a substance as aforesaid; or
(*c*) any invention capable of being used as or as part of a surgical or curative device,

the comptroller shall, on application made to him by any person interested, order the grant to the applicant of a licence under the patent on such terms as he thinks fit, unless it appears to him that there are good reasons for refusing the application.

(2) In settling the terms of licences under this section the comptroller shall endeavour to secure that food, medicines, and surgical and curative devices shall be available to the public at the lowest prices consistent with the patentees' deriving a reasonable advantage from their patent rights.

(3) A licence granted under this section shall entitle the licensee to make, use, exercise and vend the invention as a food or medicine, or for the purposes of the production of food or medicine or as or as part of a surgical or curative device, but for no other purposes.

The main points to be noted about this section are

(i) The mandatory wording—"the comptroller shall . . . unless he sees good reasons to the contrary".
(ii) "Any person interested" can apply.
(iii) The comptroller is required, in settling the terms of the licence, to secure that the public can obtain the substance at the lowest price consistent with the patentee's deriving a reasonable advantage.

It is generally considered that a licence under this section would be granted to any reputable firm who filed an application with the intention of manufacturing the patented product as distinct from merely importing it.

The section was first enacted by the Patents and Designs Act, 1919. It arose from the realisation during the 1914–18 war that Britian had become dependent on other countries, especially Germany, for many essential drugs, and it was thought that if compulsory licences were obtainable fairly easily that dependence would cease.

Medical Inventions

In the report of the Departmental Committee which preceded the Patents Act, 1932, it was stated[68] that the provision for giving the inventor "due reward for the research leading to the invention"—the wording in 1919, modified in 1949 to that quoted above—had met with the strongest criticism "as recognising and sanctioning the principle of deriving private gain from the patenting of medical inventions". This criticism came from the medical profession. One can appreciate and up to a point admire the attitude, but its effect in practice has been most unfortunate. As was pointed out in evidence to the Sargant Committee[69] "British industry and research were being handicapped in that the results of British investigations were being exploited by foreigners who had not the same objections to medical patenting as the British medical worker". No doubt there will be some here this morning who can recall, as I can, instances where a medical man has been reluctant to conduct clinical trials on a substance made by a patented process, and if this attitude was still adopted it could have an even worse effect on industrial research than the exploiting by foreigners of British inventions. The Sargant Committee recorded that they felt strongly that any ethical code enforced by medical men among themselves "should not operate to discourage that full co-operation between laboratory and clinical investigations which is essential to progress in this important field of human welfare"[70].

The evidence about the patenting of medical discoveries which was submitted to this Committee included a memorandum from the Medical Research Council which can be taken as expressing the views of at least the higher ranks of the medical profession at that time. The M.R.C. recognised that patenting of medicines might be desirable in order to exercise control over the application of the invention or to prevent improper exploitation. In accordance with this view they had accepted the patent rights covering the manufacture of insulin so as to be able to control its strength and quality. This control became unnecessary when the Therapeutic Substances Act, 1925, was passed, and the patent had been allowed to lapse. The M.R.C. considered that such cases were unlikely to recur in future.

The memorandum expressed disapproval of the action of Prof. Steenbock in obtaining patent protection for the method of making vitamin D, the disapproval being based not so much on an objection to medical patents as on the argument that the invention owed a great deal to the prior work of others (including M.R.C. workers), a comment which is true of most inventions. The Council's conclusion was that it was desirable to "secure either the total abolition of the right of patenting in the medical field, or some nearly equivalent restriction of that right". They claimed that patenting medical discoveries did not stimulate research because the incentives to research were other than pecuniary. Further, they claimed that "the Patent Law here works mischievously, because of the undue advantage obtainable by the few, mainly foreigners, who resort to it". As a further reflection of the pre-war attitude to patents in the medical field I may remind you that compounds of which the

processes of manufacture were the subject of patent rights were not included in the British Pharmacopoeia (except if the patent rights were due to expire within a short time of publication) until the issue of 1948.

This attitude to medical patents was not very different from that of other official bodies and academic workers to patents in general. It was war-time experience that led government departments, and eventually others, to realise that it was not in the national interest to make free gifts of British inventions to manufacturers in other countries. Accordingly the Development of Inventions Act, 1948, provided for the establishment of the National Research Development Corporation, the object of which is to secure the development and exploitation of inventions resulting mainly from public research, such as that carried out in government research establishments, universities and hospitals, and by the M.R.C. In 1950 the Council of the British Medical Association endorsed the report of a special committee which stated that there was no longer any objection to patenting inventions made by members of the medical profession provided the patents were assigned to the N.R.D.C.[71] The acceptance by non-industrial workers of the principle of patenting their inventions has been accompanied by a gradual but now complete disappearance of the former reluctance on the part of medical men to co-operate with industry in conducting clinical trials merely because the products to be tested were patented.

Penicillin

Perhaps the greatest single cause of a change of front on the part of the medical profession was the case of penicillin as its seems to be commonly understood. Penicillin is frequently regarded as a British discovery which was exploited in America because the discoverers did not protect their invention by patents. Sir Howard Florey has been reported[79] as regretting his "failure to patent the drug". At that time, as I have already mentioned, product patents were not obtainable in this country. A product patent would have been obtainable in U.S.A. but it seems doubtful whether a patent for the product known as penicillin in 1940 would have covered the products developed later such as the alkali metal salts of benzylpenicillin. According to the report just mentioned Florey said "If the process of extracting penicillin had been patented it would have saved me a good deal of worry in subsequent years. It seems to me that only by having available funds obtained by this means is it possible in Great Britain at the present time to be sure of keeping tried research workers and providing with certainty the income, security and facilities which first-rate people in their thirties are surely entitled to".

Processes of manufacture could of course have been patented, but again it would not have been possible, in any specification drafted before Florey and Heatley went to America to interest manufacturers there in the new drug, to have forestalled the innumerable patents relating to deep fermentation and extraction which those manufacturers afterwards obtained.

Although therefore I do not think Florey would have been able to save himself as much worry as he appears to believe, the second sentence of the above quotation presents in striking simplicity the whole case for a patent system for medicines as for any other products. He draws attention to what anyone in industry, but unfortunately not always elsehwere, would regard as self-evident fact. Unless a product resulting from industrial research can be protected, at least for a time, so that the firm concerned can recoup itself for the cost of research, it will not be able to conduct research in future. The public recognition of this by so eminent and experienced a member of the medical profession as Florey shows how great is the change in the attitude of the leaders of the profession during the 30 years that have elapsed since the M.R.C. gave evidence to the Sargant Committee.

The case for retaining the right to acquire patents for medicines is precisely the same as that for having a patent system at all. The object in either case is to stimulate new manufacture. If medicines are regarded as being of greater importance than other items considered to be essential in civilised communities, the incentive to produce new medicines should be made greater, not less. This point was well expressed in the report of the Sargant Committee[73]:

"We fully recognise . . . the *prima facie* desirability that any important invention in the medical field should be available as speedily and freely as possible for the relief of human suffering. But a corresponding importance attaches to the encouragement of industry and invention for the purpose of discovering methods of alleviating this suffering. And if, in general, the disadvantages of the monopolies granted by a patent system are more than counter-balanced by increased stimulation of industry and invention we see no reason for thinking that the same result should not equally obtain in this particular field".

On this view there is no justification for the inclusion in our patent law of the provision for compulsory licensing under patents for food and medicines. The reason for its inclusion is emotional rather than logical—the fear that the sick (and that includes us all at one time or another) will be held to ransom by some wicked patentee who will demand our health or our money. The safeguard against this possibility seems to lie in the provisions for controlling abuse of monopoly in general, provisions which seem to be accepted as adequate when other essential goods are involved. As mentioned above a compulsory licence under any patent can be obtained when "a demand for the patented article in the United Kingdom is not being met on reasonable terms"[74]. It is no doubt difficult to decide what is "reasonable" in regard to prices, but even if a Court adopted what a patentee would regard as a more liberal attitude than that taken by a politician concerned with the cost of the N.H.S., the public would seem to be adequately protected so far as the law can do so. It is a matter for speculation how the interpretation of "reasonable" in this section would compare with that in the section relating to the compulsory licensing of patents for medicines, which requires that the public shall be able to obtain the medicines at the lowest

prices consistent with the patentees' deriving a reasonable advantage from their patent rights.

It should be mentioned that an application for a compulsory licence under a patent relating to food or medicines can be made as soon as the patent is granted[75], whereas under the abuse of monopoly provisions the application cannot be made for 3 years so as to give the patentee time to organise himself.

Although the compulsory licensing provision for medicines has been part of British patent law for 40 years, it has been invoked on only two occasions, apart from one case[76] where an applicant intended merely to import a food ingredient covered by a patent, and on appeal from the Comptroller's decision the application was refused. In the first case[77], the product concerned was vitamin B_1 and a licence was granted with a royalty of $7\frac{1}{2}$ per cent of the net invoice price. In the second[78], which concerned chloramphenicol, the Comptroller invited the parties to make an agreed suggestion as to what the royalty should be.

A variety of reasons have been advanced to explain why the section has not been used more frequently. Among those mentioned to the Swan Committee[79] were the fear of retaliatory action by the patentee in respect of patents owned by the licensee, a feeling of uncertainty as to the principles upon which the Comptroller would exercise his powers, and the fact that the product made under licence could not be sold under the trade mark used for the original product, and would therefore have to compete initially under the handicap of an unknown proprietary name.

To these must be added the very unconvincing reason advanced by a member of Parliament a few months ago[80] that "many manufacturers are quite unaware of the existence of the opportunity afforded by this branch of legislation". This is certainly untrue so far as the medium and large firms are concerned, and if perhaps it applied to one or two of the smaller ones it is fairly certain that any firm who is unaware of the provision would not have the knowledge and resources to exploit it. As I am in what is, I think, the unique position of having been concerned in both the compulsory licence applications that have been made I think I can suggest that while a licensee's inability to use the established trade mark is one of the difficulties which must necessarily be taken into account, it is merely one aspect of a much broader commercial problem. The question that must be answered before a decision is reached as to whether to apply for a licence is simply "Can the product be made and sold profitably?" A licence will not bring any "know-how". This has to be acquired the hard way and it is impossible to foresee the time it will take. Even if cost estimates look promising when compared with the patentees' existing selling prices, an allowance of unknown magnitude must be made for price reduction. But the prospective applicant's real problem arises from the fact that the licence will be effective only in the U.K. Most of the firms with patents likely to be the subject of applications of this kind will be of substantial size and their business conducted on a world-wide scale. The patentee will be supplying the requirements of this country from a plant which will be large enough to

supply the requirements of several other countries as well and he will therefore have the great advantage in production costs that goes with large scale manufacture. The licensee must be able to compete after paying a royalty and in spite of the fact that he can make the product only on the scale required to supply a part of the U.K. market. Although his available market is so limited he will have all the headaches inseparable from making a new product and selling it under a new trade name—and some account must be taken of the irritation of both chemist and doctor at having another brand of the product to stock and another name to remember. It seems obvious that in the absence of special circumstances a manufacturer will prefer to devote his energies to the development of his own products which he can sell in all his markets at home and overseas.

Although a compulsory licence has so rarely been applied for, it is of course possible that the existence of the provision has had the effect of making patentees more ready to grant licences voluntarily in the belief that more favourable terms could be negotiated with the licensee than would be fixed by the Comptroller. There is no means of knowing whether there has been any such effect.

It will be realised that the reasons which, I suggest, have resulted in only two compulsory licence applications having been made in 40 years apply also to the safeguards against abuse of monopoly. The law provides the machinery, but only the industry can apply it and it is only if there is the grossest abuse that it is a practicable proposition to do so.

The whole subject of compulsory licensing in regard to medicines seems to have suffered from confused thinking. The position may be described as follows. Monopolies in general are contrary to the public interest but so far as they provide an incentive to introduce new manufactures they are a good thing, and so we have patents. But an inventor may patent something really useful which the public come to regard as essential and exploit his invention in a way which is contrary to the public interest, so we have protective provisions against abuse of monopoly. Medicines, however, are so exceptionally important to the public as compared with other essential goods that the inventor of a medicine, even if he does not abuse his monopoly, should share the benefit of his successful research work with other firms in return for a royalty, although of course those other firms are not called upon to share the cost of his unsuccessful research work. The other firms must not import the product but must go to a good deal of trouble to find out how to make it and must undertake quite substantial commercial risks; the law cannot help in these matters so the original patentee has a sporting chance of maintaining his monopoly.

We need to develop a clear conception of where the balance of public interest really lies. I may remind you of the recent report of the Hinchliffe Committee[81] in which it is said "Firms should be encouraged to increase their research effort. The conditions which favour profits for research, such as patent rights . . . should be accepted". On the one hand, therefore, is the desirability of stimulating research on the widest possible scale, on the other hand there are safeguards against abuse of monopoly.

Between these two there seems no logical place for any special arrangements for medicines, or indeed for anything else. If compulsory licences were available under all patents, the effect would be to make it more profitable to let the other fellow spend money on research and then to copy his product and pay him a royalty—unless of course he was able to keep his invention secret and not patent it.

It is submitted that the logical conclusion is that this type of compulsory licence should be abolished and that we should rely on the abuse of monopoly provisions in the same way as we do in the case of patents outside the medical field. Perhaps those provisions need reconsideration —for example it might be advantageous to reduce the 3 years delay before they can be applied. But I invite you to consider whether it should not be a requirement of the law that there should be some act or default on the part of the patentee, contrary to the public interest, before he can be required to share with competitors the results of his research work. At present, all that is required is that he should have patented a meritorious invention.

In their Second Interim Report the Swan Committee accepted the logic of the situation and recommended the abolition of the special compulsory licensing provision for food and medicines[82]. In their Final Report[83], however, they seemed again to adopt the emotional rather than the logical approach. Having argued themselves into a recommendation that chemical substances in general should be patentable as such, and that for the sake of uniformity no exception should be made for substances used for food or medicine, they said that their recommendation could be "safely" adopted if the compulsory licensing provision were retained, and their previous recommendation was withdrawn.

Medicinal Patents Overseas

While therefore there is no clear line of policy in this country as to what the public interest really requires it may be some consolation that other countries treat patents for medicines in a different way from patents for other things.

Italy has the unique distinction among the large manufacturing countries of the Western world of not allowing patents for medicines. The absence of patents for medicines has made it possible for the Italian pharmaceutical industry to make a number of important drugs evolved in industrial research laboratories in other countries. No doubt the ability to make these compounds without incurring the cost of research has played a significant part in re-establishing the Italian industry since the war. The apparent paradox that in Italy industry has been helped by the absence of patents, whereas normally industry is helped by the grant of patents, is of course explained by the fact that Italy is the odd man out. An Italian manufacturer can make and sell products which are patented in other countries not only in Italy but also in territories where the inventor has not sought patent protection and even in countries where patent applications are pending, although he may have to withdraw from the market when the patent is granted. At the same time, in

countries other than his own, he can enjoy the benefits of patent protection for his own inventions.

The absence of patents for medicines in Italy has been the subject of numerous commercial and diplomatic representations from many countries especially America and Switzerland, but so far without success. It is to be expected that the position will change when the Italian industry is sufficiently well established to originate new products and wishes to protect them at home as well as in foreign countries. Another situation can occur which may lead the Italian pharmaceutical industry to accept the re-introduction of patents for medicines. This is where a firm enters into an agreement on a royalty basis to make the product of a foreign inventor who provides the requisite know-how and then another firm works out how to make the product. Not having to pay royalty the latter can compete successfully with the licensee. If and when Italy does take patents for medicines into her system it is probable that the fear of being dependent on foreign sources for essential medicines will lead to the adoption of compulsory licensing and if that is done the British industry, at least, will not be in a position to complain.

In France, patents have been obtainable for processes for manufacturing medical products, except when the product was identifiable only by reference to the process so that the grant of a patent for the process would have given the patentee a monopoly of the product. Apart from this, a partial monopoly of a new medicinal substance has been possible by means of the visa system, but product patents for pharmaceutical compositions and medicines were forbidden as long ago as 1844.

This position was altered by an Ordinance published last February[84] which rescinded the prohibition of product patents in the medical field, although procedural details have yet to be announced.

An explanatory statement[85] declared that the ordinary patent system could not be applied to medical products without modification; production, quality and price, it was said, could not be left to normal market mechanisms. Provision was therefore made for the grant of compulsory licences if a French or foreign inventor delays manufacture or manufactures on too small a scale, or if his product is unsatisfactory in quality or price—in other words if the monopoly conferred by the patent is abused.

In the official statement I have mentioned the view was expressed that the compulsory licensing provision would rarely be used because the possibility that a licence might be granted would be sufficient to bring the patentee to a better realisation of his responsibilities. It was also claimed that the change in the law would have the following advantages—the inventor would have proper protection, research would be encouraged, multiplication of identical products would be prevented, and the international repute of France would be enhanced by the termination of a system which has enabled French industry to copy foreign inventions. These are large claims. Perhaps the most interesting having regard to the fact that in Italy not even processes are patentable in the medical field, is the reference to the international repute of France. In view of

what I have said about the British compulsory licensing provisions it is also interesting to note that France considers compulsory licences should be granted only if the patent monopoly is abused.

In continental countries in general, chemical processes can be patented but not the products, and chemicals having therapeutic properties are treated in the same way as others. In most South American countries, new chemical compounds can be patented as well as the processes of making them, but a distinction is made in the case of medicines so that that only the processes of making them can be protected.

In Canada substances made by chemical processes and intended for food or medicine cannot be claimed as such, except when prepared by the method described in the patent specification[86], but there is no bar on patents for therapeutic compositions not made by chemical processes. While, therefore one cannot obtain a patent for a new drug itself however made, one can patent a pharmaceutical preparation of the drug and thus obtain virtually all the protection that would be given by a patent on the drug.

In America, as perhaps one would expect, the full commercial logic of the matter is applied and a new drug or process for making it can be patented in the same way as any other chemical. There seems to be no suggestion there that the public would gain by weakening the patent monopoly of a medicine by the grant of compulsory licences, neither is there any worry about abuse of monopoly by a single manufacturer—and it would certainly be difficult to demonstrate by reference to the American pharmaceutical industry that patents do not provide a stimulus to the discovery of new drugs.

Commonwealth countries have tended to follow the United Kingdom practice of providing for compulsory licences, but so far as I can ascertain the provision has not been used. In India the matter is under review and the authorities have been seeking the views of the pharmaceutical industry.

Of 18,450 complete specifications accepted by the Patent Office in 1958, more than 2,000 were classified as being primarily concerned with chemical processes or compounds[87]. No figures are available to show how many of these were in the pharmaceutical field but it is certain that pharmaceutical manufacturers who undertake research are making considerable use of the patent system. In this highly specialised field I cannot claim any status other than that of a keenly interested amateur, but it is my hope that this review will have shed some light on a branch of law which to a greater extent than we may realise effects us all both as pharmacists and as citizens.

References

1. Senkus *v.* Johnston, *United States Patent Quarterly* (U.S.P.Q.), 77, 113, at p. 115.
2. See article by Gordon Grant, Comptroller-General, *Board of Trade J.*, 1959, **176**, 1181.
3. G.E.C.'s Application, 60 R.P.C. 1.
4. Lenard's Application, 71 R.P.C. 190.
5. Bovingdon and I.C.I.'s Application, 64 R.P.C. 20.

6. Canterbury Agricultural College's Application, 1958 R.P.C. 85.
7. Patents Act, 1949, section 14 (1) (*d*). (Subsequent references when only the number of a section is given relate to the Patents Act, 1949.)
8. Section 32 (1) (*e*).
9. Section 50 (1).
10. See reference 2.
11. Samuel Parkes & Co. Ltd. *v.* Cocker Bros. Ltd., 46 R.P.C. 241, at p. 248.
12. Section 14 (1) (*e*).
13. Section 32 (1) (*f*).
14. See para. 72 *et seq.* and Note of Dissenting Members, Second Interim Report of the Departmental Committee on the Patents and Designs Act, Cmd. 6789, 1946. This Committee is referred to subsequently as "the Swan Committee".
15. May & Baker Ltd. and Ciba Ltd.'s Patent, 65 R.P.C. 255, at p. 281.
16. Section 32 (1) (*g*).
17. Section 4 (1)
18. Section 4 (3)
19. Application for Revocation of May & Baker's Patent, 66 R.P.C. 8.
20. R. F. Haslam, *Trans. Chart. Inst. Pat. Agents*, **75**, C41, at p. C46–47.
21. 35 United States Code (U.S.C.), 102 (*b*).
22. 35 U.S.C. 135.
23. See G. H. Naimark, *Drug & Cosmetic Industries*, 1958, **82**, 596.
24. 35 U.S.C. 101.
25. 35 U.S.C. 112.
26. *In re* Bremner, Taylor & Jones, 86 U.S.P.Q., 74.
27. Article 1, section 8.
28. Blicke *v.* Treves, 112 U.S.P.Q., 472.
29. *Ex parte* Tolkmith, 102 U.S.P.Q. 464.
30. *In re* Oberweger, 47 U.S.P.Q. 455.
31. *In re* Nelson and Shabica (Private communication; report not yet available in U.K.).
32. *Ex parte* Posnack & Michaels, 103 U.S.P.Q. 64.
33. *In re* Isenstead *v.* Watson, 115 U.S.P.Q. 408.
34. *Ex parte* Ferguson, 117 U.S.P.Q. 229.
35. Hans Dersin, *Gewerblicher Rectsschutz und Urheberrecht*, 1958, **60**, 413.
36. Section 4 (1).
37. Rule 38 (1), Patents Rules, 1958; S.I. 1958, No. 73.
38. Section 13 (4).
39. Viscount Simonds in Patchett *v.* Sterling Engineering Co. Ltd., 72 R.P.C. 50, at p. 56.
40. Section 56 (1).
41. Section 56 (2).
42. Marshall and Naylor's Patent, 17 R.P.C. 553.
43. Section 4 (5); and Rule 11 (i), S.I. 1958, No. 73.
44. Patents and Designs Act, 1919, section 38A (1).
45. M's Application, 39 R.P.C. 261.
46. Memorandum, Part II, of the Committee, submitted to the Board of Trade Patents Committee, 1944, para. 55.
47. Final Report, Cmd. 7206, 1947, para. 95.
48. British Patent No. 712,948.
49. Section 10 (1) (*c*).
50. Section 4 (7).
51. 22nd May, 1959.
52. British Pharmacopoeia 1958, p. XI.
53. Section 35.
54. 19 U.S.C. 1337a.
55. *N.R.D.C. Bulletin*, April, 1958, 8.
56. Section 32 (1).
57. *Inventions, Patents and Monopoly*, by P. Meinhardt, 2nd Edn., Stevens Bros. Ltd., London, 1950, p. 177.
58. Restrictive Trade Practices Act, 1956, section 8 (4).
59. Section 57.
60. Macleans Ltd. *v.* Craven Park Cut Price Stores, reported in *Pharm J.*, 1939, **89** (4th series), 209.
61. 35 U.S.C. 271 (*c*).
62. Section 65.
63. Sections 37 to 39.

64. Section 37 (2).
65. Section 39.
66. Second Interim Report, para. 24.
67. Section 41.
68. Report of the Departmental Committee on the Patents and Designs Act and Practice of the Patent Office, Cmd. 3829, H.M.S.O., 1931, paragraph 188. This Committee is referred to subsequently as the Sargant Committee.
69. Idem, paragraph 189.
70. Idem, paragraph 200.
71. *Brit. med. J. Suppl.*, 1956, **1**, 176.
72. *Chem. & ind.*, 1958, p. 1412 (Oct. 25th).
73. Paragraph 199.
74. Section 37 (2) (*b*).
75. Parke Davis & Co. *v.* The Comptroller-General, etc., 71 R.P.C. 169.
76. Schou's Patents, 41 R.P.C. 298.
77. Glaxo Laboratories Ltd.'s Application, 58 R.P.C. 12.
78. Unreported.
79. Second Interim Report, paragraphs 37 to 40.
80. See *Pharm. J.*, 1959, **182**, 133.
81. Final Report of the Committee on the Cost of Prescribing, H.M.S.O., 1959, para. 258.
82. Paragraph 65.
83. Paragraphs 97 to 99.
84. Ordonnance No. 59–250, dated 4th February, 1959; *J. Officiel de la Republique Française*, 1959, pp. 1756–59.
85. Idem.
86. Canadian Patents Act, 1935, section 41.
87. Report of the Comptroller-General for the year 1958. H.M.S.O., 1959.
88. Mond Nickel Co. Ltd.'s Application, 1956, R.P.C. 189.

SYMPOSIUM

THE MODIFICATION OF THE DURATION OF DRUG ACTION

PHARMACOLOGICAL AND CLINICAL CONSIDERATIONS

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THE usual reason for attempting to modify the actions of a drug is to increase its usefulness in the treatment of disease. Sometimes, however, the purpose may be to improve its prophylactic value, or occasionally to provide a more rapid, precise or safer diagnostic agent. Drug action can be modified in a number of ways; by altering its physical properties by adding to it other substances or other drugs, or by modifying its chemical structure.

In considering the modification of drug action for therapeutic purposes, it would be generally true to say that the aim of any kind of treatment is to control the disease process or the symptoms to which it gives rise in the shortest period of time and with the least inconvenience to the patient. The success or failure of modifying the action of a drug can therefore be gauged by whether the patients who receive treatment with the modified drug are cured or relieved of their symptoms more rapidly, more completely, or more frequently than they would with the unmodified drug. There are several important points to observe in making comparisons of this kind but I do not propose to discuss them in detail. I would like to emphasise however the necessity to distinguish pharmacological effects from the effects of suggestion. This is not to underestimate the value of therapeutic suggestion, but rather to point out the danger of drawing the wrong sort of conclusions. For example, if one were to accept all that has been written about new methods of improving drug action, it would seem that no one need suffer headache for more than 3 minutes or lie awake counting sheep—or overdrafts—for more than 5 minutes. The rapid effect obtained after swallowing a capsule would seem to be connected more with the act of taking the capsule rather than with the action of its contents.

Whatever measurements are chosen as an index of pharmacological action, no valid comparison of drug action can be made without them. They can serve to distinguish between a scientific advancement and a sales-promotion gimmick. The nature of the comparison will of course depend on the particular circumstances. It may consist in measuring the relative concentration of the drugs in the blood, cerebrospinal fluid or urine, or in determining by some biochemical tests, the changes produced on the blood sugar level, on the electrolytes excreted in the urine, or in the case of some antibiotics, the antibacterial activity of the serum or urine. Simple clinical measurement of changes in blood pressure, pulse rate, body weight or body temperature may be sufficient or more complicated assessments may be considered necessary, such as measurement of

cardiac output, vital capacity, retention of iodide, differential blood cell counts or the number of parasites killed or eliminated.

The apparatus required may be complex and expensive, or on the other hand, a pen or pencil may suffice to record how many times a patient coughs, how long he sleeps or how frequently or to what extent he has relief of pain.

Clinical Considerations

Modification of drug action may be required in order to provide (*a*) a more rapid therapeutic effect; (*b*) a more prolonged and sustained action with less frequent administration; or (*c*) a safer or less disturbing therapeutic effect less liable to be accompanied by other actions or side-effects of the drug.

Increasing the Onset of Action

Many of the drugs which are absorbed from the alimentary tract are reasonably well absorbed within 15 to 45 minutes of oral administration, and their action can be expected well within this time range. For many patients this is a convenient and satisfactory form of treatment. There are occasions however where, because of the severity of illness or because the patient is unable to swallow or retain substances given by mouth, some more rapid or alternative method of treatment is necessary. This can often be achieved by temporarily changing the route of administration of sulphonamides or of tetracycline drugs, for example, in the initial stages of treating severe acute infections. Herein lies the importance of providing sterile preparations of the latter suitable for intravenous as well as for intramuscular injection.

Sometimes the onset of action of a drug normally administered by intramuscular or subcutaneous injection is delayed by the presence of gross oedema or adipose tissue, or on account of peripheral vascular failure. Here again a change to intravenous injection, for example, of mersalyl, insulin or of an analgesic such as morphine provides a satisfactory answer to the problem.

The use of hyaluronidase to increase the rate of absorption by increasing the subcutaneous dispersal of fluid, is a valuable therapeutic method of rapidly restoring water and electrolyte balance especially in infants and young children. The advantages afforded by the use of a spreading factor have also been readily appreciated by surgeons and anaesthetists to increase the rate of action of local anaesthetics, though some care must be exercised in controlling the spread of effect and also the resultant decrease in duration of local anaesthesia.

The rapid absorption of iron by intramuscular injection of iron-dextran complex is a notable contribution which might be expected to stimulate further work in this field. For example, a non-irritant intramuscular preparation of aminophylline would be a welcome alternative to intravenous injection—at present the only effective method of giving this valuable drug.

There appears to be a considerable increase in the number of drugs which are prepared for parenteral administration. In the United States

of America during 1957, a total of 496 new products and preparations were introduced of which 52 were for parenteral administration, and 36 more were made available in 1958[1]. Some of these consist of hormones such as gonadotrophins, corticotrophin and oxytocin which can only be administered by injection, others such as antispasmodics, antihistamines, tranquillising drugs and vitamins are more difficult to justify on the basis of a need to increase the speed of action or to facilitate administration.

More use could be made of the sublingual route of administration. The success of nitroglycerin and isoprenaline tablets for the treatment of angina and bronchospasm is well recognised. The rapid relief of symptoms and the ease with which the effective dose can be quickly determined, specially commend this method for emergency treatment. It could with advantage be extended to the study of potent analgesic and cough suppressant drugs. The same could be said of administration of drugs in aerosols.

Prolonging Duration of Action

There are several reasons for attempting to prolong the action of a drug, the most important of which is to maintain the therapeutic effect for longer periods than can be obtained after administration of a single dose. Moreoever this might be expected to diminish the waxing and waning of drug action following frequent administration of single doses. Other reasons such as saving the time of the nursing staff, more convenience for the patient, or avoiding reliance on his memory are probably less important and less convincing.

There are a number of outstanding examples where this method of modifying the action of a drug has been successful, notably in the treatment of certain endocrine diseases such as diabetes mellitus, diabetes insipidus, Addison's disease and related conditions requiring supplemental therapy with adrenal cortex hormones. It is significant that in all these instances the drug has been administered parenterally, usually because of its inactivation or inadequate absorption in the gastrointestinal tract.

These therapeutic successes have encouraged the extension of this type of modified preparation to other drugs, the successful outcome of which is more doubtful. There are however many clinical indications where the sustained or prolonged action of a drug is necessary. In the treatment of infections, adequate and continuous action of the chemotherapeutic agent is vital even though the therapeutic programme is restricted to 7 or 10 days. The argument is even greater for treatment with antituberculosis or antileprotic drugs which are required to be given for many months. This also applies to the treatment of a number of tropical diseases and helminthic infections.

Continuous therapy is also necessary in the management of peptic ulceration, which demands continued action of the drug throughout the night and early morning. Antacid or antispasmodic drugs with a prolonged action would therefore be very acceptable additions to the drugs at present available. Longer-acting ganglion blocking and tranquillising

drugs would be useful in the treatment of hypertension, and similar modifications of solanaceous alkaloids and of antihistamine drugs would be valuable in maintaining symptomatic control of Parkinsonism and of some allergic diseases. Severe continuous or intermittent pain is a distressing symptom of a number of diseases and longer acting analgesic drugs would be gladly welcomed. A notable exception to this is the treatment of angina of effort with nitrites where to continuously obscure the warning symptoms of pain, and therefore of over-exertion, would most likely do more harm than good.

The effective symptomatic control of cough is appreciated by the patient and just as often by those in his immediate environment. There is much to be said therefore for the provision of a long acting cough suppressant drug. According to one statement[2] there is now available a preparation containing 5 mg. of dihydrocodeinone, a single dose of which will stop a useless cough for 6000 jet-miles. How many miles have to be travelled before the effect begins may perhaps best be answered by the fellow-travellers.

The success which follows attempts to prolong the action of a drug must be judged by whether the modified drug produces an adequate effect for the desired period without undue delay; this last point is important but not so vital as the others, since if necessary the action can be initiated by administration of the unmodified drug.

Apart from the systemic administration of drugs some attempts have been made to prolong the action of those which are intended only for local administration. The most notable example is the use of adrenaline as a vasoconstrictor to delay absorption and prolong the action of local anaesthetic drugs. Many instances of ischaemic necrosis have resulted from the use both in medical and dental practice of high concentrations of adrenaline. It is noteworthy that there is now fairly general agreement amongst clinicians that for this purpose, the maximum effective concentration consistent with safety is a 1 in 100,000 (0·001 per cent) solution of adrenaline. The use of compressed pellets of hydrocortisone for the treatment of ulcers in the buccal cavity focuses attention on the importance of exploring further this method of prolonging the local action of drugs.

Pharmacological Considerations

Intensity and duration of action depend on the concentration of drug on the cells on which it acts and this is determined by its rate of absorption, distribution to and clearance from the tissues. Thus the action of a drug may be prolonged by reducing its rate of absorption, by delaying the rate at which it is inactivated, or if it is excreted unchanged, by retarding its excretion.

In general the most practical and successful methods depend on reducing the rate of absorption and, because of the greater number of variable factors which influence absorption from the gastrointestinal tract, modification of drugs for parenteral administration have produced more satisfactory results than those for oral administration.

Parenteral Administration

The more water-soluble the drug the more rapidly it is absorbed; therefore absorption will be delayed when the drug is administered in a relatively water-insoluble form. This can be done by changing the solvent to an oily vehicle or by adding to the aqueous solution a colloid such as gelatin, polyvinylpyrrolidone or dextran. The former has been useful in the earlier days of administering penicillin, vasopressin, and a number of other drugs, but absorption is erratic and the oily vehicle complicates the cleaning of syringes. The use of gelatin has been effective in delaying absorption of corticotrophin but is not reliable for the administration of heparin. Some anxiety has been expressed about the carcinogenic potentialities of polyvinylpyrrolidone but this should not be allowed to preclude its use for short-term therapy, or where the patient already is the victim of malignant disease. It would be interesting to know whether a long acting potent analgesic can be formulated with this substance. More evidence would be relevant regarding the use of dextran in prolonging the action of ganglion-blocking drugs, neostigmine, heparin and analgesic drugs.

These and other methods of delaying absorption such as forming water insoluble salts, esters or protein complexes will be discussed in detail by Dr. Edkins. It is relevant to point out that they apply mainly to drugs such as insulin, corticotrophin, heparin and penicillin which are fairly rapidly cleared from the body. No useful measure is served in extending investigations of this kind to drugs, for example, thyroxine, digitalis glycosides or cyanocobalamin, which though required continuously, are themselves slowly eliminated.

The action of some drugs, notably the stable steroid hormones, can be prolonged by preparing them as compressed or fused pellets. Three methods of administration are being increasingly and successfully explored in medical, dental and veterinary practice. Subcutaneous implantation of DOCA pellets for the treatment of Addison's disease is one of the classical examples; much more frequent use is made of this method of administering oestrogens and androgens. Sublingual absorption of methyl testosterone is another use, and more recently the prolonged local action of hydrocortisone in the buccal cavity. Oral administration of pellets has also promising application in veterinary practice. These different methods of administering pellets doubtless pose a number of problems in formulation.

Oral Administration

So far as I am aware, the present fashion for taking medicine only once a day has not spread to the taking of food and I would like to express the hope that the idea of squeezing the most out of a drug, by allowing it to be absorbed slowly and preventing any wasteful excretion of it, does not invade or threaten the pleasure of eating three square meals a day. Until much more is known about the laws that govern the absorption of drugs from the gastrointestinal tract and their subsequent utilisation, there is little prospect of introducing successful automation of the alimentary tract.

There is no harm is trying out these ideas with drugs that don't matter very much, but as Dragstedt[3] has emphasised they should not be applied at present to potent drugs such as digitalis glycosides, antibiotics and ganglion blocking drugs, precise and adequate doses of which are vital if they are to contribute to the wellbeing and life of the patient.

At one time it was considered that ethyl alcohol was the only drug that was effectively absorbed from the stomach, but there is increasing evidence that the stomach is an important site of absorption of drugs. Various factors however influence the extent to which this occurs. One is the ability of the drug to dissolve in the gastric contents; for example, a moderately soluble compound like aspirin is readily absorbed, whilst a poorly soluble one such as dicoumarol is not. Hogben and his colleagues[4] have found that if a drug is partially unionised in the acid gastric contents it is well absorbed, whereas little is absorbed if it is ionised. This is attributed to the fact that the gastric mucosa acts as a lipid barrier which selectively permits passive diffusion of the unionised lipid-soluble form of a drug but prevents the diffusion of the lipid-insoluble ionic form. For example, organic acids such as aspirin and barbiturates with a pKa of 3 or more which are undissociated in acid gastric juice are rapidly absorbed, whilst bases such as quinine, ephedrine and amidopyrine with a pKa greater than 5, which are ionised are not absorbed. Phenazone, a weaker base which in the presence of gastric secretion is not dissociated, is quite well absorbed.

A similar type of pattern seems to condition absorption from the intestine where there is a fairly close relation between the lipid solubility of unionised drug and the rate of intestinal absorption[5]. The idea of a lipid barrier does not satisfy all the requirements for absorption of a drug because it does not account for the passage of inorganic ions and other lipid-insoluble substances which are required for cell metabolism. Doubtless other special transport mechanisms are involved.

There are of course other factors which determine the extent to which drugs are absorbed from the stomach. If a drug is taken with water before a meal, it may remain only for a short time and little of it is absorbed from the stomach. If it is swallowed soon after a meal, the delayed emptying favours absorption but the rate at which this occurs is delayed by the volume of the gastric contents.

Individual variation in the amount of drug absorbed is another important factor; some individuals are relatively poor absorbers. This is common experience but is illustrated by the evidence recently reported by Hogben and his colleagues[4]. Observations were made on three healthy adults of the amount of drug absorbed after its administration by gastric tube. After 20 minutes the amounts of aspirin absorbed by each of the subjects expressed as a percentage of the dose were 31, 42 and 16 respectively.

The phenomenon of individual variation not only in respect to the rate and extent of absorption but also in relation to the response to drugs emphasises the importance of paying due attention to the individual needs of the patient and severely restricts the likelihood of regulating drug therapy by alarm-clock methods of sustained release.

There are two examples in veterinary medicine which illustrate the successful modifications of drug action on the alimentary tract. The first concerns the use of the "cobalt bullet". Cobalt deficiency in ruminants gives rise to a deficiency in the production of vitamin B_{12} by microorganisms in the rumen. Thus in certain areas of Southern Australia when sheep and cattle are grazed on pasture consisting predominantly of *Phalaris tuberosa*, the animals frequently develop a demyelinating disease, Phalaris staggers, which is often fatal. This can be prevented by oral administration of cobalt which promotes an adequate biosynthesis of vitamin B_{12}. Dewey, Lee and Marston[6] have given a fascinating account of how they succeeded in preparing compressed cylindrical pellets containing cobaltic oxide in china clay. The specific gravity of the pellets was such that when swallowed they were transported fairly rapidly to the reticulum, where because of their density, they remained and from them cobalt was released for many weeks. This report is important because it illustrates how a careful study of drug absorption can be carried out both *in vitro* and *in vivo* and it also provides the basis for investigating the prolonged administration of other substances such as anthelminthics to ruminants.

The other example concerns the anthelminthic phenothiazine. Kingsbury[7] and others[8–10] have demonstrated that in the treatment of nematode infections of sheep the particle size of this water-insoluble compound is an important factor in determining its anthelminthic activity and hence its therapeutic efficiency. Extensive studies by these investigators have shown that the most active preparations were those which were prepared from phenothiazine containing a high proportion of particles of 10μ or less. This work illustrates the importance of determining the physical properties of a drug which are necessary for its optimum local action, consistent with low absorption.

Delaying Inactivation

Apart from altering its chemical structure there are few practical methods of modifying the rate of inactivation of a drug. One notable example is the inhibition of enzymes involved in the inactivation of drugs. The action and uses of anticholinesterase compounds such as neostigmine depend on their ability to combine with cholinesterase and prolong the action of acetylcholine by delaying its hydrolysis. Various attempts have been made to increase and prolong the action of other drugs, for example morphine, by concurrent use of one of these inhibitors[11].

A new approach to this aspect of prolonging drug action is the discovery of some compounds which by themselves have little or no action but which are able to prolong the action of a variety of other drugs by inhibiting the enzyme systems in liver microsomes which inactivate them[12,13]. β-Diethylaminoethyl diphenylpropylacetate (SKF525–A), 2,4-dichloro-6-phenylphenoxyethyl diethylamine (Lilly 18947), and isopropyl-2-isonicotinyl hydrazine (iproniazid, Marsilid) have been shown to interfere with the metabolism of barbiturates, amphetamine, acetanilide, pethidine and amidopyrine. These substances have not yet been investigated

sufficiently to establish their therapeutic value but their potential uses are considerable.

There are several examples of drugs whose actions can be prolonged by modification of their chemical structure. The different rates of inactivation of barbiturates provide a selection of compounds varying in duration from two or three hours (quinalbarbitone) to 12 hours or more (phenobarbitone). An anticholinesterase compound can be chosen to provide for diagnostic use the brief action of edrophonium, or for therapeutic purposes the longer action of neostigmine or pyridostigmine. A large series of similarly acting organophosphorus compounds consisting amongst other of dyflos, tetra-ethylpyrophosphate and octamethyl pyrophosphate (OMPA) can produce even more prolonged action but the difficulties of controlling the extent of enzyme inhibition within safe limits have severely restricted their general therapeutic uses.

A formidable amount of information has been collected on the outstanding value of many of these compounds as insecticides[14]. Some of those with low toxicity in mammals, notably malathion and chlorthion, have been formulated as dusting powders for the control of human body louse infections. The beneficial results to the community at large, which have accrued from the careful systematic and coordinated researches on this aspect of drug action are indeed noteworthy.

Delayed Excretion

One of the classical methods of prolonging drug action is the curtailment of salt whilst administering bromides. It has been known for many years that the therapeutic benefit of bromide depends on establishing an optimum concentration of it and maintaining this by balancing the amount absorbed and excreted. This drug has been largely discarded but I hope the wisdom of knowing how to use it remains. The excretion of drugs, such as penicillin and aminosalicylate, which are eliminated by tubular excretion can be delayed by concurrent administration of caronamide or probenecid which interfere with transport mechanisms in the renal tubules. The practical problem of conveniently maintaining an effective concentration of the interfering drug has severely restricted the therapeutic usefulness of this method.

The action of some drugs may be prolonged on account of their storage in tissues from which they are slowly released and excreted. This property may be important in the choice of a particular drug from several which have similar pharmacological actions. The fact that chloroquine, after oral administration, is highly concentrated in the liver from which it is gradually released makes it specially valuable for the prophylactic control of malaria. The accumulation of chlorotrianisene (TACE) in the body fat has led to the use of this drug as a long-acting orally administered oestrogen[15,16].

Reducing the Side Effects

One of the most important therapeutic tasks is the problem of choosing from a number of drugs with similar actions the one which is likely to be

most suitable for the individual patient. It is a problem which arises from the fact that each patient, in a sense, is a law unto himself and that his response and reactions to drugs cannot be accurately predicted. It is common knowledge that the dose of a drug which is necessary to produce its typical effect in a group of individuals may vary over a four-fold range or more. Because of this wide individual variation in response, many potent and therefore potentially toxic drugs must be administered in such a way that the dose can be adjusted according to the individual need and response. Most experienced clinicians adopt this procedure when prescribing treatment with well established drugs such as digitalis, insulin, salicylates and morphine. Unfortunately this principle seems to be overlooked when newer drugs are used. Thus when a new drug is tried in the recommended dose with little or no therapeutic effect, or if its effect is accompanied by nausea, headache or some other uncomfortable side-effect, instead of adjusting the dose or frequency of administration treatment is too often abandoned in favour of some other drug or mixture of drugs.

This is one of the chief reasons why mixtures of drugs in one preparation have become so popular. A critical examination of many or these however reveals no evidence of any rational pharmacological basis for therapy. The virtue of some appears to lie in the fact that the dose of each constituent is sufficiently small not to give rise to any serious risk of overdose, nor indeed to any significant pharmacological action. Perhaps there is some substance in the remark of the cynic who advocated a policy of basing his therapy on 90 per cent suggestion and 10 per cent pharmacology.

The major criticism of preparations containing mixtures of anti-histamine and amphetamine drugs, amphetamine and barbiturates, antispasmodic and antacid drugs and many others of this nature is the fact that the fixed proportion does not readily permit any adjustment of the dose of each constituent drug. Preparations containing an active drug and one of its antagonists have little to commend them.

A notable exception to these is the use of three sulphonamides instead of one but with the synthesis of less toxic sulphonamides, this may be regarded more as of historical interest.

Hypersensitivity reactions to drugs scarcely come within the province of this discussion. The fact that some individuals respond in a wholly abnormal manner to normal or subnormal doses of a drug should always be anticipated, and when it occurs should be noted and respected by both patient and physician. This applies not only to reactions to penicillin, sulphonamides, barbiturates and many other new drugs, but just as much to aspirin. The only safe modification is to change the drug rather than the patient.

One of the best reasons for encouraging attempts to reach the moon is not so much for what is likely to be found there, but because a considerable increase in the amount of knowledge will be gained in the process of reaching this objective. The same can be said of many investigations which have been pursued on the theme of modifying drug action. This is

meant as a tribute to the ingenuity of pharmaceutical formulation, but it is intended also to convey a plea for a more realistic appreciation of the pharmacological limits which condition such manipulations of drugs.

References

1. De Haen, *The Bulletin of the Parenteral Drug Association*, 1959, **13**, 3.
2. *Medical Science*, 1959, **5**, 825.
3. Dragstedt, *J. Amer. med. Ass.*, 1958, **168**, 1652.
4. Hogben, Schauker, Tocco and Brodie, *J. Pharmacol.*, 1957, **120**, 540.
5. Hogben, Tocco, Brodie and Schauker, *ibid.*, 1959, **125**, 275.
6. Dewey, Lee and Marston, *Nature, Lond.*, 1958, **181**, 1367.
7. Kingsbury, *Vet. Rec.*, 1958, **70**, 523.
8. Douglas, Baker and Longhurst, *Amer. J. vet. Res.*, 1956, **17**, 318.
9. Gordon, *Austr. Vet. J.*, 1956, **32**, 258.
10. Whitten, *N.Z. Vet. J.*, 1956, **4**, 63.
11. Christensen and Gross, *J. Amer. med. Ass.*, 1948, **137**, 594.
12. Brodie, *J. Pharm. Pharmacol.*, 1956, **8**, 1.
13. Fouts and Brodie, *J. Pharmacol.*, 1955, **115**, 68.
14. Negherbon, *Handbook of Toxicology*, Vol. 3, *Insecticides*. W. B. Saunders Co., Philadelphia, 1959.
15. Thompson and Werner, *Proc. Soc. exp. Biol. N.Y.*, 1951, **77**, 494.
16. Greenblatt and Brown, *Amer. J. Obst. and Gynec.*, 1952, **63**, 1361.

THE MODIFICATION OF THE DURATION OF DRUG ACTION

Pharmaceutical Considerations

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Factors Modifying the Rate of Onset of Action of a Drug

If the title of this Symposium is interpreted in its most rigid sense it could be argued that modification of the time of onset of the action of a drug is excluded from consideration. Nevertheless, immediate onset of action is nearly always desirable both in normal dosage forms and in prolonged acting preparations. Occasionally, delayed onset is deliberately arranged, as in one brand of an hypnotic tablet which is claimed to contain an "alarm clock" which acts some 8 hours after ingestion, but in practice the differences between individuals to which Dragstedt[1] has drawn attention are so great that it is doubtful if a preparation of this nature is of value.

Oral preparations. In unmodified oral preparations, speedy onset of action is dependant upon rapid disintegration and it is doubtful, even now, whether its importance is fully appreciated. In various countries the permitted disintegration times of Official tablets differ widely; the British Pharmacopoeia with a few exceptions requires 15 minutes as a maximum, while the United States Pharmacopoeia specifies times varying from 15 minutes to 2 hours or even longer.

The disintegration time found for a particular tablet will vary according to the method employed for its determination which is usually specified by the Pharmacopoeia concerned. The use of a moving disc in the tube holding the tablet, permitted by the British Pharmacopoeia[2] if the tablets do not otherwise comply with the official requirements, and now obligatory in the Unites States Pharmacopoeia[3] usually decreases disintegration time, but with some tablets made with a base which becomes sticky when wet and which stick to the disc, this time is substantially increased.

Although a maximum disintegration time of 15 minutes is the lowest requirement of any Pharmacopoeia, it is possible to prepare tablets which disintegrate more rapidly and this is particularly desirable in preparations given to produce direct relief, such as analgesics and symptomatic anti-asthmatics, and drugs intended to combat conditions such as migraine.

A number of factors influence the disintegration rate of tablets; composition, the disintegrant and the manner in which it is incorporated, the lubricant, the water-content of the granule and compression. The most generally used disintegrant is maize or potato starch, but Berry and Ridout[4] drew the attention of this Conference in 1950 to the value of alginic acid, a claimed advantage being that since it may be wetted and re-dried without loss of swelling and therefore of disintegrating power, it may be used as the granulating agent by adding it to the tablet mix after allowing it to swell in water, so that the granules themselves contain a disintegrant. If they do not it is possible for a tablet to pass the disintegration test of both the British and United States Pharmacopoeias by

disintegrating to granules which pass the 10 mesh wire sieve specified, while the granules themselves may disintegrate only slowly with consequent slow release of the medicament. Tablets prepared by direct compression of a water soluble substance when it occurs in a suitable granular or crystalline form should also contain a disintegrant, for tablets of sodium chloride, sodium bromide or calcium gluconate, for example, dissolve slowly unless disintegration is assisted by a suitable additive.

Hydrophobic lubricants such as magnesium stearate or talc delay disintegration and though often more effective than starch the use of the latter has the advantage that it assists, rather than impedes, disintegration.

The disintegration time of tablets may change on storage; usually to lengthen though occasionally it becomes shorter. This is associated with a change in hardness, and when the disintegration time lessens the tablets sometimes become so soft that they are no longer usable. This change is often associated with the water content of the granules at the time of compression. Granules compressed with a water content in excess of their equilibrium value with normal atmospheric humidity produce tablets which on storage lose moisture and become harder with a corresponding increase in disintegration time, the reverse being also true. While the former condition is the more common, the increasing practice of air-conditioning, when the relative humidity is held below normal, has led to the use of starch to control the moisture content and of glycerol to prevent excessive drying[5]. Compression at the time of manufacture is of importance; excessive compression leading directly to increase in disintegration time.

Parenteral preparations. More immediate onset of action usually follows parenteral injection, particularly by the intravenous route. This may involve no change in the drug itself as with Vitamins B_1 and C, a number of alkaloids and other substances normally administered orally. Slight change in the chemical nature can frequently render suitable for injection drugs which orally are given in an insoluble form, thus the sodium salt of prednisolone hemisuccinate is soluble in water and may be given intravenously, by which route it exerts a spectacularly instantaneous effect when compared with the oral use of prednisolone, the intramuscular injection of a suspension of the steroid or even with the infusion of an equivalent dose of Hydrocortisone Injection, while phenobarbitone as the sodium salt given intravenously acts very rapidly. Physiologically, the simultaneous use of hyaluronidase hastens the absorption of drugs given subcutaneously or intramuscularly.

Prolongation of Drug Action

Oral Preparations

Lazarus and Cooper[6] have summarised the aims which oral sustained release preparations have been designed to accomplish as follows.

"To provide rapid onset of activity by immediate release of an amount of the active ingredient sufficient to raise the level of the drug in the body to a therapeutic optimum".

"To maintain a steady therapeutic drug concentration".

"To eliminate deficiency in concentration due to divided or improperly spaced doses".

"To reduce the number of doses administered".

"To lessen the hazard of defaulting from prescribed treatment by reducing the frequency of dosage".

Dragstedt[1] earlier suggested that praiseworthy as objectives such as these may be, they are not necessarily always achieved nor should it be assumed that they constitute encouragement to the extension of such preparations in a random or wide-spread manner. He draws attention to the number of variables involved when a tablet is taken orally, insofar as the time the tablet remains in the stomach and intestine, and the nature of the digestive juices with which it comes in contact, are concerned, and he considers that it would be valueless to attempt to determine the average of such variables which would apply to only a few individuals. He states that no drug should be given in the form of a prolonged action preparation if accuracy of dosage is important, if absorption from the gastrointestinal tract is impaired or erratic or if the total dose administered as a prolonged action preparation is more than two or three times the usual therapeutic dose unless the drug concerned is known to have a wide margin of safety. Campbell and his colleagues[7–10] have studied the "physiological availability" of certain drugs, particularly those where absorption could be measured by their concentration in the blood and urine, as a function of the disintegration time of the tablets containing them and from this stated that simple tablets should have an *in vitro* disintegration time of less than 60 minutes to ensure complete availability of the drug. This is not confirmed by all workers. Tablets of sodium salicylate, the disintegration of which has been delayed by enteric coating, exert satisfactory activity[11], and Lazarus and Cooper[6], quote evidence, mainly obtained with the sulphonamides which can be readily determined in body fluids and tissues, which has shown that prolonged acting preparations do provide physiologically significant blood levels even when the disintegration time of the dosage form is greater than 60 minutes.

Methods which have been used to gain some picture of the release rate of a drug from a prolonged-action preparation *in vivo* when the determination of its concentration in blood is impossible have been mainly confined to following the disintegration of tablets containing a radio-opaque substance by X-rays, though this has limited application in man due to possible damage to the tissues. This method has been used to follow the disintegration rate of barium sulphate capsules *in vivo*[12] the time taken being about double that found by the USP method, and this suggests that the values for the release rate of a drug from a prolonged-action preparation found *in vitro* can be used only with caution when referred to the release rate to be expected in man.

In vitro methods of determining release rates are described by Lazarus and Cooper[6]. The procedures, which differ mainly in detail dependant upon the type of preparation being tested, normally employ simulated

gastric and intestinal juices at 37°, controlled agitation of the eluant and of the preparation being tested and some type of sieve to separate the disintegrated particles from the bulk of the product. In all cases the amount of drug released at varying time intervals is calculated from the assay of either the eluant or the residue remaining undisintegrated.

Pharmaceutical Methods of Prolonging Drug Action in Oral Preparations

(*a*) *Tablets and capsules.* An early attempt to provide a repeat action involved the simultaneous ingestion of two tablets or capsules one of which had received a coating intended to delay availability of the drug for some hours, by which time it was assumed that the effect produced by the normal preparation would have disappeared. This method was quickly simplified by preparing a tablet made up of a core containing one dose coated with a delaying layer covered with a layer containing an initial dose on the outside. This does not provide the continuous slow release of medicament which is desired and is no better, but only more convenient, than taking two tablets at an interval of time. It is also difficult in practice to control the manufacturing process of pan coating so that a uniform thickness of coat is obtained and very variable disintegration times have been reported[13] for commercial batches of enteric coated tablets, even within the same batch.

A considerable advance in obtaining continuous slow release is due to Blythe[14] who, instead of a "repeat action" tablet, suggested the use of a large number of small pellets coated with varying thicknesses of a coat intended to delay the release of the drug, some pellets being left uncoated to provide the initial dose, the mixed pellets being supplied in hard gelatin capsules. These pellets are manufactured by applying the drug to a core, commonly sugar granules, and then coating by the pan coating process, coatings of different thicknesses being distinguished by colour. The lack of uniformity in coating thickness associated with this process is here an advantage, since with the large number of pellets in each capsule it is reasonable to assume that continuous slow and regular release of the drug will be achieved.

Tablets have been prepared either from a mixed granulate, part of which has been treated to retard disintegration or from coated pellets using a wax or fatty base to prevent damage to the protective coating on compression, but scoring or deformity of the coating may occur during tabletting causing variation in the rate of release of the drug[6].

(*b*) *Oral preparations other than tablets and capsules.* Though most work has been carried out on tablets and capsules for prolonged action medication the application of this principle to liquid preparations has not been entirely overlooked. Lang[15] has summarised the methods now in use. A water-insoluble drug may be prepared as a suspension either using material of large particle size, or after adsorption to an ion exchange resin or protein. The drug may also be suspended in a water-in-oil emulsion, or coated with substances insoluble in the gastric juice. Methods of preparation are quoted by Lazarus and Cooper.

Chemical Methods of Prolonging Drug Action in Oral Preparations

The most fully exploited chemical method to obtain slow release in oral preparations involves combination of an acidic or alkaline drug with an ion exchange resin[16,17]. Alkaline drugs combined with cation exchangers of the sulphonic acid type to give a resinate, release the drug as the hydrochloride in both stomach and intestine and since elution is dependent only upon ion concentration[18] which does not vary widely in the digestive juices the release rate is fairly constant.

A more regular rate of elution is obtained if incompletely converted resin, or a mixture of the resinate with the hydrogen form, is used, since this depresses the initial release rate. Acidic drugs combined with an anion exchanger release the drug in the acidic form in the stomach and as the sodium salt in the intestine, but have not yet been widely used.

Other chemical methods which have been employed for obtaining prolonged action in oral preparations involve either change in the chemical nature of the drug itself or the manufacture of derivatives. The barbiturate series is an example of the former and exhibits the common characteristic of such modifications that usually the interval between administration of the drug and the onset of its therapeutic effect corresponds to its duration of action.

Examples of the latter are methylpentynol and mephenesin which, as the carbamates, have a lengthened duration of action.

Physiological Methods of Prolonging Drug Action in Oral Preparations

In certain instances the action of drugs given orally can be prolonged by simultaneous administration of a substance which competes with them for excretion by the renal tubules. Caronamide[19,20], sodium benzoate[21] and probenecid[22,23] have been used for this purpose.

A number of subtances are now under clinical investigation which delay or inhibit drug metabolism. Of these, aminoethyldiphenylpropyl acetate and 2,4-dichloro-6-phenylphenyloxyethyldiethylamine have been claimed greatly to prolong the action of phenytoin and methoin, morphine and pethidine, and amphetamine.

Parenteral preparations. Parenteral preparations having prolonged activity are of particular importance for medicaments which are rapidly eliminated and require frequent administration if a satisfactory level in blood and tissues is to be obtained, and for those drugs which have to be given for a long time or throughout the lifetime of the patient. For this reason they have been largely confined to the field of penicillin and the hormones, in the former case because of the necessity of obtaining rapidly a high concentration of the antibiotic in the blood and maintaining this high level during treatment in the face of rapid deactivation and excretion, and in the latter to enable a chronic condition to be treated for a long time without the necessity of frequent injections.

The methods which have been employed to increase the duration of activity of parenteral preparations may be summarised as follows. 1. Physiological modification of absorption or excretion. 2. Modification

of the solvent. 3. Chemical modification of the drug. 4. Adsorption of the drug. 5. The use of suspensions. 6. Implantation pellets.

This classification is somewhat idealised for it is very common for several of these methods to be employed simultaneously to obtain the desired result.

Physiological modification of absorption or excretion. It is well known that a drug given by the intramuscular or subcutaneous route is less rapidly effective than the same drug given intravenously. Increased duration of action may follow modification of the route of injection, thus heparin given intravenously increases blood coagulation time within about 10 minutes but the effect is transient, usually having disappeared within about 3 hours, while the same preparation given in larger dose intramuscularly produces a less rapid response but a substantially prolonged effect[24]. Absorption can be delayed by adding to the injection a vasoconstrictor such as adrenaline, as in procaine—adrenaline. Attempts have been made to extend this principle to insulin and to penicillin[25], but it is unsuitable for continuous treatment as the number of injections of adrenaline involved may lead to secondary effects. Caronamide and probenecid given by mouth, delay the excretion of certain antibiotics, particularly penicillin, given simultaneously by injection, but it is unlikely that such a method can ever be of general application.

Modification of the solvent. When a drug is soluble both in water and in an oily vehicle, as, for instance, with oestrone in low concentration, the oily solution is significantly more long acting than the aqueous one. For water-soluble drugs, the addition of gelatin, carboxymethylcellulose and polyvinylpyrrolidone to the aqueous injection has been claimed to increase the duration of activity of some[26–28] and to be ineffective with others[29,30] and the use of carboxymethylcellulose and polyvinylpyrrolidone is declining following warnings that they may be carcinogenic in the high concentrations required[31,32] Dextran which is claimed to be non-carcinogenic[32] has been suggested as an alternative.

It is doubtful whether the increase of viscosity of the solution caused by the presence of the colloid is responsible for the delay mechanism, though increased duration of action is shown by procaine penicillin suspensions sufficiently concentrated to have thixotropic properties[33]. It has been suggested that apart from complex formation[34–36] the delay action of polyvinylpyrrolidone may be due to the formation of a micelle into which the medicament is adsorbed or to a slowing of renal excretion without apparent disturbance of the renal function, particularly as it is effective parenterally in association with a drug given orally[26], but at the present time the mechanism is by no means clear.

Addition of beeswax to a suspension of penicillin in oil[37] causes substantial prolongation of action though it has been suggested that the mode of action may be due rather to the production of a mild inflammatory reaction in the tissues than to the increased viscosity of the injection. Beeswax may be replaced by aluminium monostearate in penicillin in oil injections and is effective in prolonging the effect of oil solutions of cyanocobalamin[30].

The action of a water-soluble drug may be prolonged by preparing a water-in-oil emulsion of the aqueous solution, and a heparin preparation of this type has been suggested[38].

Chemical modification of the medicament. Chemical modification is directed to either the production of insolubility or delay in utilisation. The former is well exemplified by penicillin and insulin. The combination of procaine and benzyl penicillin yields procaine benzylpenicillin[39] which is only slightly soluble in water and in oils and which, in suspension in oil, is long acting both *in vitro* and *in vivo*[40]. Numerous other insoluble salts of penicillin have been the subject of investigation and patents. The advantages of such derivatives are three-fold, firstly the insolubility of the product in water confers stability, rendering possible the formulation of aqueous suspensions which retain their activity for a long time; secondly, on injection, the insoluble material forms a depot from which the derivative is slowly leached, and thirdly there is probably some further delay in utilisation while the derivative is broken down in the body to release an active penicillin molecule. Esters of penicillin do not appear to be effective in man[41,42], owing to the absence of a penicillin esterase in human blood which is present in some animal species[43].

Insoluble compounds of insulin were introduced with the intention of modifying the transitory nature of soluble insulin. The relatively insoluble complex of protamine and insulin[44] first produced in 1936 was shortly afterwards superseded by protamine zinc insulin[45] the extreme insolubility of which confers remarkable stability and an increase in the duration of activity.

Other proteins may be similarly employed, the prolonged action being less pronounced when a protein less alkaline than protamine, which has an isoelectric point of about 12, is used.

Delayed action preparations such as isophane or NPH insulin containing relatively little zinc and a reduced amount of protamine have a shorter duration of action than protamine zinc insulin[46] but have the advantage that they may be mixed with unmodified insulin for simultaneous injection when the quick action of the latter is required, whereas with protamine zinc insulin in similar conditions the rapid action of the added unmodified insulin is lost.

Sensitivity may be encountered with these preparations due to the protein content, and more recently insoluble zinc-insulin preparations free from protein have been made[47] by precipitating insulin in the presence of zinc under nearly neutral conditions in the absence of ions having an affinity for zinc, giving either amorphous or crystalline material which may be suspended in an aqueous vehicle for injection; the amorphous material is rapid in onset of action which is prolonged for about 12 hours, while the crystalline material is rather slower in onset and lasts about as long as protamine zinc insulin. The great difference between the particle sizes of the amorphous and crystalline types does not lead to the difference in duration of activity which might be expected, which suggests that the variation between the two types is more fundamental than this.

MODIFICATION OF THE DURATION OF DRUG ACTION

Chemical modification of the steroid hormones has not been directed towards the production of insolubility since this group is without exception insoluble in water at a therapeutic dosage level, but has been concerned with delaying metabolism to an active molecule. Esterification of one or more of the free hydroxyl groups in the steroid molecule by an organic acid is the method usually adopted and Junkmann and Witzel[48] have listed some 500 steroid esters most of which have been tested pharmacologically for prolongation of activity, the greatest amount of work having

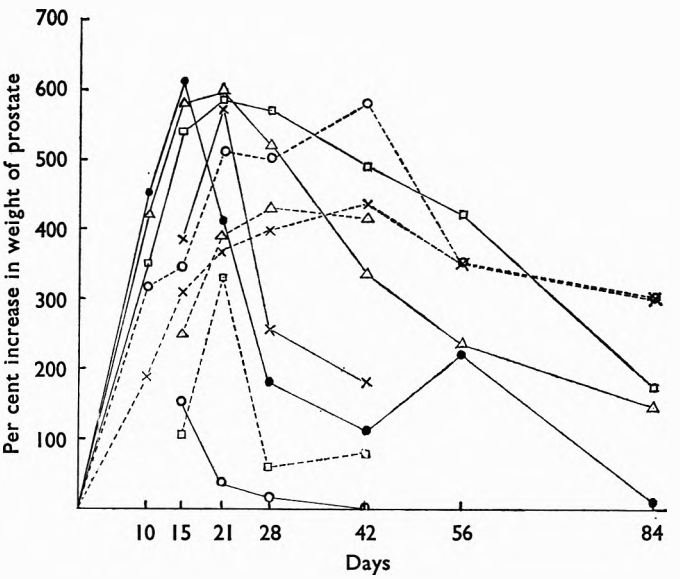


FIG. 1. Percentage increase in prostate weight of castrated rats after a single intramuscular injection of a series of testosterone esters. Figures in parentheses show the number of carbon atoms in the esterifying acid.

○——○ propionate (2)
△——△ oenanthate (7);
○---○ pelargonate (9);
△---△ undecanoate (11);
●——● phenylpropionate for comparison.
X——X caproate (6);
□——□ caprylate (8);
X---X decanoate (10);
□---□ laurate (12);

(After Dekanski and Chapman, unpublished.)

been directed to the esters of the androgens and, in particular, testosterone. Reasonably good agreement has been found in this series between the duration of activity and the rate of saponification of the ester treated with homogenised human or rat liver[49], the lower the rate of hydrolysis, the longer acting the ester, but Junkmann and Witzel point out that similar agreement was not obtained, particularly with testosterone phenylacetate, phenylpropionate and hexahydrobenzoate, when the rate of saponification was determined in alcoholic potassium hydroxide[50].

In general, esterification with a long chain fatty acid produces the greatest prolongation of activity, which increases to a maximum with an optimum chain length of the acid. Figure 1 shows the result of some

unpublished work carried out in our own Laboratories on a series of esters, varying in chain length from C_6 to C_{12} which suggests that C_9 (pelargonate) or C_{10} (decanoate) is the optimum for this series. The initial delay in response characteristic of most long acting preparations is also seen and demonstrates that if a uniform level of activity with rapid onset is to be obtained a mixture of esters such as the propionate, phenylpropionate and decanoate should be used, rather than the long acting decanoate alone. These esters become more soluble in oil with increase in chain length of the esterifying acid, enabling the larger doses required to be given in a small dose-volume.

Adsorption. Adsorption of a drug on to an insoluble carrier has been used as a means of prolonging the activity of toxins and toxoids, and is of considerable importance as one method of delaying the utilisation of the protein hormone, corticotrophin. The carrier is usually the hydroxide or phosphate of either aluminium or zinc, adsorption occurring only when these substances are in a colloidal form.

In 1954, Homan and others[51] showed that both zinc phosphate and zinc hydroxide, precipitated from a solution of corticotrophin, carried with them the corticotrophic activity and produced a suspension of small particle size which was easily injected and from which the active material was slowly released after injection; they considered also that since zinc can inhibit several kinds of protein splitting enzymes[52–54] the simultaneous slow release of zinc compounds present in this preparation would protect the corticotrophin from destruction.

Preparations based on zinc phosphate are clinically effective[55] but may crystallise on storage with release of adsorbed corticotrophin into solution and a consequent decrease in the duration of activity. Suspensions are freshly prepared for clinical use by mixing a solution of corticotrophin containing zinc chloride with a solution of sodium phosphate containing sodium hydroxide before injection. A similar preparation using zinc hydroxide, which is not liable to crystallise, as the carrier, is manufactured ready for use.

Crystal suspensions. Crystal suspensions of steroid hormones are not as long acting as might at first be considered probable. A crystal suspension of testosterone propionate is intermediate in duration of effect between the oil solution and an implantation pellet[56] and while pharmacological work has shown that both duration and response is about doubled when the crystal suspension is compared with the oil solution of this steroid ester[57], the response to the crystal suspension declines rapidly. The duration of action of such crystal suspensions varies directly with the size of the crystals[58], but a limit must be imposed since pain on injection increases with increasing crystal size[59]. Crystal growth occurs with some steroids and while it can be largely prevented in many cases by careful formulation, it is potentiated by continuous fluctuation of temperature which should be avoided during storage. Attempts have been made to overcome these difficulties by dissolving the hormone in a water-soluble solvent, which, on injection, should dissolve in the body fluids leaving a crystalline residue of the steroid *in situ*; propylene glycol solutions have

been used for this purpose, and a more elaborate method consists in dissolving the hormone in benzyl alcohol which is soluble about 1 in 25 in water and emulsifying this solution in water saturated with benzyl alcohol[60]. Some suspensions are still widely used not because of any great prolongation effect but because solubility considerations render this the only way to provide a therapeutic dose in a reasonable dose-volume.

Suspensions prepared from steroids crystallised under non-sterile conditions, when spores may act as centres of crystallisation, are difficult to sterilise by autoclaving unless the particle size is less than 50μ[61], and this finding is of importance since the normal test for sterility would not indicate the presence of viable spores within the crystals.

Implants. The form of prolonged duration medication introduced in 1937[62] originally known as implantation pellets and now officially abbreviated to "implants", consists of a hard tablet which, inserted beneath the skin or into the muscle tissue, slowly dissolves in the body fluids which surround it, liberating continuously a small amount of the medicament into the blood stream.

A medicament in this form must be active in very small dosage, and must be required to exert its activity for long periods, and for these reasons its application has so far been limited to the restricted but important field of hormone therapy where it closely simulates the effect of a functioning gland.

Attempts have been made to prepare implants of water soluble hormones by mixing the drug with insoluble substances such as cholesterol before compression[63] but all implants at present available are prepared from one or other of the steroid hormones, which are just sufficiently soluble in body fluids to enable a therapeutic level to be maintained.

Implants are manufactured either by compression of the pure sterile material, or by melting and casting into shape in a mould. The former method is of universal application, but the latter is restricted to hormones with a melting point below about 200°, since those with a higher melting point discolour or char on melting, and is not satisfactory for the stilboestrol series of synthetic oestrogens which fall to powder after solidification. Little difference has been noted clinically in the effect of implants prepared by either method though it is claimed that the process of fusion leads to a more uniform product than can be obtained by the compression process. Protein matter deposits within the substance of a compressed implant[64] after insertion, and it has been suggested[65] that this is due to the presence of pores in the compressed product which are presumed to be absent in that made by fusion. This deposition may be demonstrated by dissolving the implant, after removal from the body, in an organic solvent, when an insoluble residue remains in the shape of the implant to which the name "ghost" has been applied[64], and it has been claimed[66,67] that this "ghost" formation decreases the rate of absorption. A capsule of connective tissue also forms in the body around implants of either type but this is not deleterious. In spite of the presumed less porous structure of fused implants there is evidence that they tend to be utilised more rapidly than those made by compression[65].

The mobilisation of the hormone is limited by the rate of solubility in the body fluid rather than by the final solubility of the substance. The absorption rate from superficial areas can be expressed mathematically as a hyperbolic curve[65] which reflects the decreasing surface area of the

TABLE I

CORRELATION OF APPROXIMATE AVERAGE DAILY ABSORPTION RATE AND SURFACE AREA IN FUSED IMPLANTS OF DEOXYCORTONE ACETATE

Weight (mg.)	Diameter (mm.)	Length (mm.)	Surface area sq. mm.	Average daily absorption (mg.)	Average absorption mg./sq. mm.
25	2·2	6·0	49	0·30	0·0061
50	4·5	2·8	71	0·45	0·0063
100	4·5	5·5	110	0·70	0·0063
150	4·5	8·3	149	0·90	0·0060
200	4·5	11·0	187	1·10	0·0058
300	7·0	6·6	222	1·30	0·0058

implant during absorption and we have found that if the average daily absorption is related to the original surface area, fair agreement is seen (Tables I and II).

This consideration is of importance in controlling, not only the daily dosage but also the duration of activity. Increased dosage rate can be achieved most accurately by increasing the number of implants inserted at one time, whereas an increase in the weight of material implanted as a single

TABLE II

CORRELATION OF APPROXIMATE AVERAGE DAILY ABSORPTION RATE AND SURFACE AREA IN FUSED IMPLANTS OF TESTOSTERONE

Weight (mg.)	Diameter (mm.)	Length (mm.)	Surface area sq. mm.	Average daily absorption (mg.)	Average absorption mg./sq. mm.
25	2·2	6·0	49	0·40	0·0081
50	4·5	2·8	71	0·70	0·0098
100	4·5	5·5	110	1·10	0·0100
150	4·5	8·3	149	1·35	0·0090
200	4·5	11·0	187	1·50	0·0080
300	7·0	6·6	222	1·70	0·0076
350	7·0	7·7	246	1·80	0·0073

pellet, while it leads to some increase in daily dosage, is of greater importance in determining the time during which the implant continues to exert its therapeutic effect.

Implants are usually prepared from the unmodified hormone if it is stable, as with testosterone, progesterone or oestradiol, or from a normal relatively short acting ester such as deoxycortone acetate if the free steroid decomposes. Use of the long-acting esters is of no advantage since the rate of utilisation is sufficiently limited by the rate of solution.

REFERENCES

1. Dragstedt, *J. Amer. med. Ass.*, 1958, **168**, 1652.
2. *British Pharmacopoeia*, 1958, p. 964.
3. Second Supplement to the *Pharmacopoeia of the United States*, 15th Rev., p. 21.

4. Berry and Ridout, *J. Pharm. Pharmacol.*, 1950, 2, 619.
5. Seth and Münzel, *Pharm. Ind.*, 1959, **21**, 9.
6. Lazarus and Cooper, *J. Pharm. Pharmacol.*, 1959, **11**, 257.
7. Chapman and Campbell, *Canad. J. Biochem.*, 1955, **33**, 753.
8. Chapman, Crisafio and Campbell, *J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 374.
9. Chapman, Chatten and Campbell, *Canad. med. Ass. J.*, 1957, **76**, 102.
10. Campbell, Chapman and Chatten, *ibid.*, 1957, **77**, 602.
11. Wruble, *J. Amer. pharm. Ass., Sci. Ed.*, 1935, **24**, 1074.
12. Feinblatt and Ferguson, *New Eng. J. Med.*, 1956, **254**, 940.
13. Kanig, *Drug Standards*, 1954, **22**, 116.
14. Blythe, *ibid.*, 1958, **26**, 1.
15. Lang, *Schweiz. Apoth. Ztg.*, 1958, **96**, 773.
16. Chaudhry and Saunders, *J. Pharm., Pharmacol.*, 1956, **8**, 975.
17. Saunders and Srivastava, *J. chem. Soc.*, 1950, 2915; 1952, 2111.
18. Abrahams and Linnell, *Lancet*, 1957, **2**, 1317.
19. Meads, Long, Pace and Harrell, *J. Amer. med. Ass.*, 1948, **138**, 874.
20. Hunter and Wilson, *Lancet*, 1948, **2**, 601.
21. Strauss, Richburg, Saba and Alexander, *J. Lab. clin. Med.*, 1947, **32**, 818.
22. Boger, Gallagher and Pitts, *J. Philadel. gen. Hosp.*, 1950, **1**, 51.
23. Boger, Pitts and Gallagher, *J. Lab. clin. Med.*, 1950, **36**, 276.
24. Abrahams, *Brit. med. J.*, 1950, **2**, 1418.
25. Fisk, Foord and Alles, *Science*, 1945, **101**, 124.
26. Durel, *J. Prat., (Paris)*, 1948, **62**, 273, 288.
27. Choay and Choay, *Ann. pharm. franç*, 1947, **5**, 420.
28. Lederer, *Acta Endocrinol.*, 1949, 2, 307.
29. Graham, Slinger and Teed, *J. Pharm. Pharmacol.*, 1954, **6**, 27, 115.
30. Arnold, *J. Amer. pharm. Ass., Pract. Ed.*, 1959, **20**, 106.
31. Hueper, *Cancer Res.*, 1956, **16**, 120.
32. Lusky and Nelson, *Fed. Proc.*, 1957, **16**, 318.
33. Ober, Vincent, Simon and Frederick, *J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 667.
34. Higuchi and Kuramoto, *ibid.*, 1954, **43**, 393, 398.
35. Higuchi and Lach, *ibid.*, 1954, **43**, 465.
36. Kennon and Higuchi, *ibid.*, 1956, **45**, 157.
37. Romansky and Rittman, *Science*, 1944, **100**, 196.
38. Jefferson, Sussman and Marder, *J. Amer. med. Ass.*, 1948, **138**, 747.
39. Sullivan, Symmes, Miller and Rhodehamel, *Science*, 1948, **107**, 169.
40. Hobby, Brown and Patelski, *Proc. Soc. exp. Biol. N.Y.*, 1948, **67**, 6.
41. Levaditi, Penau, Vaisman and Hagemann, *C.R. soc. Biol. Paris*, 1946, **140**, 737.
42. Broh-Kahn and Smith, *Proc. Soc. exp. Biol., N.Y.*, 1946, **61**, 216.
43. Ungar, *Brit. J. exp. Path.*, 1947, **28**, 88.
44. Hagedorn, Jensen, Krarup and Wodstrup, *J. Amer. med. Ass.*, 1936, **106**, 177.
45. Scott and Fisher, *J. Pharmacol.*, 1935, **55**, 206; 1936, **58**, 78.
46. Lawrence, *Brit. med. J.*, 1955, **1**, 603.
47. Hallas-Moller, Peterson and Schlichtkrull, *Science*, 1952, **116**, 394.
48. Junkmann and Witzel, *Zeit, Vit. Horm. Fermentforsch.*, 1957–8, 9, 97, 227.
49. Dirscherl and Dardanne, *Biochem. Zschr.*, 1954, **325**, 195.
50. Schenck and Junkmann, *Arch. exp. path. pharmak.*, 1955, **227**, 210.
51. Homan, Overbeek, Neutelings, Booij and van der Vies, *Lancet*, 1954, **1**, 541.
52. Bang, *Acta pharm. tox., Kbh.*, 1946, **2**, 89.
53. Adams and Smith, *J. biol. Chem.*, 1951, **191**, 651.
54. Green, Gladner, Cunningham and Neurath, *J. Amer. chem. Soc.*, 1952, **74**, 2122.
55. Ferriman, Anderson and Turner, *Lancet*, 1954, **1**, 545.
56. Freed and Greenhill, *J. clin. Endocrinol.*, 1941, **1**, 983.
57. Kupperman, Aronson, Gagliani, Parsonnet, Roberts, Silver and Postiglioni, *Acta Endocrin.*, 1954, **16**, 101.
58. Maier and Gasche, *Schweiz. med. Wschr.*, 1946, **76**, 1192.
59. Joel, *J. Clin. Endocrinol.*, 1948, **8**, 97.
60. Lens, Overbeek and Polderman, *Acta Endocr.*, 1949, **2**, 396.
61. Polderman, Bloo and Fokkens, *Pharm. Weekbl.*, 1958, **93**, 45.
62. Deanesly and Parkes, *Proc. Roy. Soc., B.*, 1937, **124**, 279.
63. Vargas, *Lancet*, 1949, **1**, 598.
64. Folley, *Nature, Lond.*, 1942, **150**, 403.
65. Bishop and Folley, *Lancet*, 1944, **1**, 434.
66. Geist, Walter and Salmon, *Proc. Soc. exp. Biol., N.Y.*, 1940, **43**, 712.
67. Walter, Geist and Salmon, *ibid.*, 1940, **44**, 314.

DISCUSSION

The following points arose out of the DISCUSSION.

While the emphasis of the meeting was on prolonging the action of drugs, extended action could be a disadvantage, as the drug might be difficult to control, and its actions impossible to terminate. There is often a need for a drug having a rapid but short duration of action. The value of a series of "peak" blood levels of a drug as opposed to "plateau" levels was a question of trial, but blood levels were only one factor. Tissue concentrations of the drug were of more importance, and diffusibility must be taken into account. Frequency of administration should be related to storage in the tissue. Prolonging action by the modification of the metabolic degradation of drugs by chemical methods was suggested, as was also chemically modifying an active compound such that upon *in vivo* attack by enzymes the parent compounds would be liberated. The information that polyvinylpyrrolidone was carcinogenic stemmed from a report of work on the substance in which the processing conditions were far more drastic than would be imposed in the preparation of medicaments incorporating the polymer, and therefore the claim was misleading. The inclusion of excipients in implants or their manufacture by the sintering process would affect the properties of the product. Evidence of the unsatisfactory nature of many enteric-coated tablets was cited. Further consideration should be given to the potentialities of sublingual absorption. Disintegration of the granules as well as of the tablets was essential for reliable absorption of insoluble drugs. Sustained action tablets should not be used for all drugs: there might be adverse effects on the kidney or liver.

SCIENCE PAPERS

Short Communication

POTENTIAL ANALGESICS. THE STEREOCHEMISTRY OF SOME ISOMERIC PIPERIDINOL DERIVATIVES

By N. J. Harper, A. H. Beckett and A. D. J. Balon

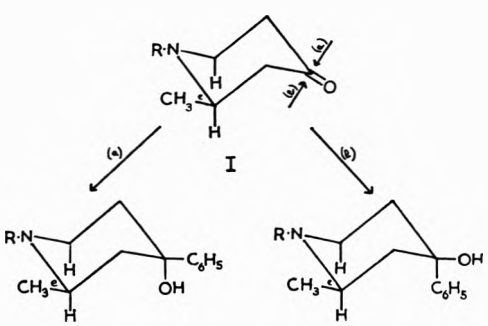
From the School of Pharmacy, Chelsea College of Science and Technology, Manresa Road, London, S.W.3

Received June 1, 1959

During continued investigations of structure-activity relationships in synthetic analgesics, some compounds related to the reversed esters of pethidine, namely isomers of *N*-(2′-phenylethyl)-2-methyl-4-phenyl-4-piperidinol, *N*-(2′-phenylethyl)-2,6-dimethyl-4-phenyl-4-piperidinol, *cis*-2,6-dimethyl-4-phenyl-4-piperidinol and some of their esters were prepared by routes involving the addition of lithium phenyl to the appropriate ketone. These compounds are of interest in view of the recent introduction by the Russians of the potent analgesic Promedol[1,2].

Treatment of *N*-(2′-phenylethyl)-2-methyl-4-piperidone (I) with lithium phenyl gave two isomeric piperidinols (Type A and B) which were separated by fractional crystallisation of the hydrochlorides. These isomers A and B (II and III) obtained in a ratio of 2:1 were assigned *cis*-CH_3/C_6H_5 and *trans*-CH_3/C_6H_5 configurations respectively on the basis of the following evidence.

(*a*) The steric course of addition of organometallic derivatives to ketones is controlled by steric hindrance involved in the approach to the carbon atom of the carbonyl group, and by an energy factor which influences the ease with which a group may be forced into an unfavoured position[3]. With *N*-(2′-phenylethyl)-2-methyl-4-piperidone, attack from side "b" is sterically hindered, and causes a preferential formation of the type A isomer (II), with an equatorial phenyl group.



R = $CH_3CH_2C_6H_5$

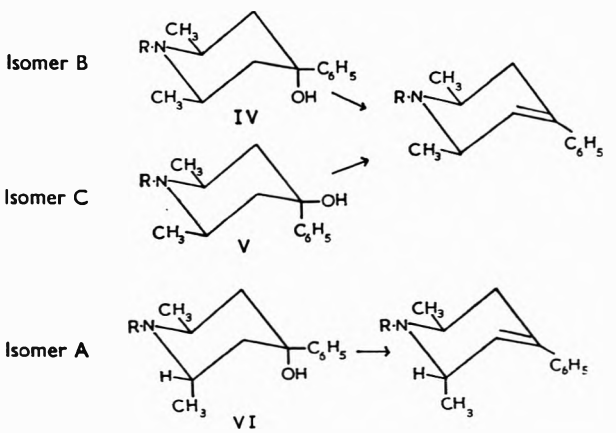
(*b*) The infra-red spectra of α and β-prodine type compounds show two regions[4,5] (Region A, 990–1010 cm.$^{-1}$ and Region B, 1350–1385 cm.$^{-1}$) which are isomerically characteristic. The infra-red spectrum of the A isomer substantially resembles those of α-prodine type compounds and that of the B isomer the spectra of β-prodine type compounds; an axial and an equatorial conformation of the hydroxyl group for the A and the B isomers respectively is indicated.

(*c*) The isomer B is more readily esterified than is A; an equatorial hydroxyl group in the former isomer is indicated.

N-(2′-Phenylethyl)-2,6-dimethyl-4-piperidone was prepared by the interaction of acetaldehyde, 2-phenylethylamine and diethyl acetonedicarboxylate. The addition of lithium phenyl to isomerically impure *N*-(2′-phenylethyl)-2,6-dimethyl-4-piperidone gave the three theoretically possible piperidinols in the ratio 9:2:1 designated Types A, B and C respectively; these were separated by fractional crystallisation of the free base.

The following configurations have been assigned. A isomer, *trans*-CH_3/CH_3 (VI); B, *cis*-CH_3/CH_3, *cis*-CH_3/C_6H_5 (IV) and C, *cis*-CH_3/CH_3, *trans*-CH_3/C_6H_5 (V), the evidence for the assignation being as follows.

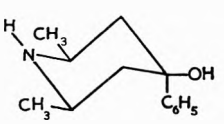
(*a*) Elimination of B and C isomers gave identical eliminated products which indicated their derivation from the *cis*-CH_3/CH_3 ketone, while isomer A gave a different eliminated product indicating its formation from *trans*-CH_3/CH_3 ketone.



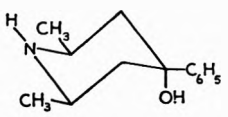
(*b*) The dissociation constants[6] of isomers B and C were 8·4 and 8·57 respectively but that of A was greatly dissimilar at 9·06; the similar geometry of the methyl groups in isomers B and C which differ from that obtaining in isomer A is indicated. An axial methyl group on a carbon adjacent to a basic nitrogen atom would be predicted to have a greater base strengthening effect than a corresponding equatorial methyl group; the *e*, *e* arrangement of the two methyl groups in isomers B and C and the *a*, *e* conformation in isomer A advanced above is therefore supported.

(*c*) Infra-red spectra demonstrated intermolecular hydrogen bonding in A and B isomers but not in C; similar arrangement of the hydroxyl groups in A and B but not in C is indicated. Isomer A with the *trans* dimethyl groups would be expected to have the CH_3 (*e*), CH_3 (*a*), C_6H_5 (*e*) and OH (*a*) conformation (VI) and isomer B is therefore given the *cis*-CH_3/CH_3, *cis*-$CH_3C_6H_5$ configuration (IV).

The reaction of *cis*-2,6-dimethyl-4-piperidone[7] with lithium phenyl gave two isomeric piperidinols, A and B, which were separated chromatographically in a ratio of 12: 13 respectively. Upon attempting to prepare hydrobromides, isomer A gave the piperidinol salt but isomer B eliminated to the tetrahydropyridine salt. Therefore the *e* and *a* conformation of the hydroxyl group in isomers A (VII) and B (VIII) respectively is indicated. This assignment is supported by esterification studies in which, using identical conditions, A but not B could be esterified. The infra-red spectra of isomer A in the 1000 to 1200 cm.$^{-1}$ region showed the strongest peak at 1141 cm.$^{-1}$, the strongest absorption of isomer B being at 1013 cm.$^{-1}$. These peak locations are in close agreement with those of isomer C (V) (1143 cm.$^{-1}$) and isomer B (IV) (1018 cm.$^{-1}$) of *N*-(2′-phenylethyl)-2,6-dimethyl-4-phenyl-4-piperidinol; the conformational similarities of isomer A (VII) to (V) and of isomer B (VIII) to (IV) is thus indicated and supports the above conclusions.



VII



VIII

The compounds and their esters are being tested as potential analgesics.

References

1. Nazarov, Raigorodskaya and Rudenko, *Izvest. Akad. Nauk. S.S.S.R. Otdel. Khim. Nauk.*, 1949, 504.
2. Nazarov, Prostakov and Shvetsov, *Zhur. Obshchei. Khim.*, 1956, **26**, 2798.
3. Berson and Greenbaum, *J. Amer. chem. Soc.*, 1958, **80**, 445.
4. Beckett, Casy and Harper, *Chem. Ind.*, 1959, 19.
5. Beckett, Casy and Kirk, *J. med. pharm. Chem.*, 1959, **1**, 37.
6. Beckett, Casy, Harper and Phillips, *J. Pharm. Pharmacol.*, 1956, **8**, 860.
7. Hall, *J. Amer. chem. Soc.*, 1957, **79**, 5444.
8. Klyne, *Progress in Stereochemistry*, Butterworth, London, 1954, Vol. 1.

After Dr. Harper presented the communication there was a DISCUSSION. The following points were made.

The acetoxy and propinoxy esters of the Isomer A of *N*-2-phenylethyl-2,6-dimethyl-4-phenyl-4-piperidinol were about four times as active as morphine when assessed by the hot plate method. In the compounds tested the various types of morphine-like activity had been clearly separated.

NEUROMUSCULAR BLOCKING AGENTS

PART IV. THE SYNTHESIS AND STUDY OF *N*- AND *S*-ALKYL VARIANTS OF DIHEXASULPHONIUM AND DIHEXAZONIUM TRIETHIODIDES

BY FIONA MACLEOD CAREY†, D. EDWARDS*, J. J. LEWIS† AND J. B. STENLAKE*

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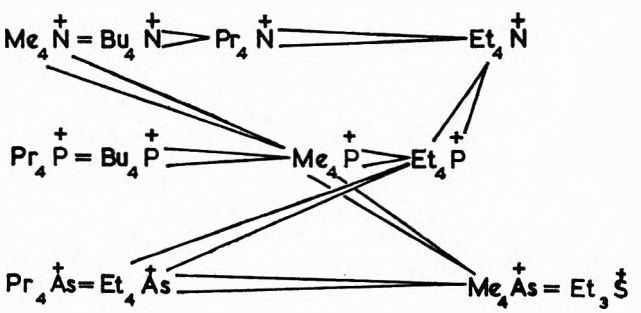
Received May 28, 1959

NSN- and *NNN*-Tris-onium compounds related to dihexasulphonium and dihexazonium in which *N*-alkyl substituents are varied have been synthesised. All compounds tested qualitatively resembled tubocurarine in their action. Stepwise replacement of Et by Me in dihexasulphonium tri-ethiodide decreases potency and some C 10-like effects appear in compounds XA and XB. Potency also falls when Et groups are replaced successively by *n*-Pr in both dihexasulphonium tri-ethiodide and dihexazonium tri-ethiodide, compounds XIF and XIG being the least active of the series and approximately equipotent with dihexasulphonium trimethiodide (XA). Replacement of one Et group by *n*-Bu at each quaternary ammonium centre of dihexazonium, as in compound XIC increases potency.

THE influence of alkyl substituents on the curariform activity of simple tetra-alkyl mono-quaternary ammonium salts has been studied on frog sartorius and gastrocnemius muscle preparations by Külz[1], Marshall[2], Peiser[3] and Ing and Wright[4]. Quantitative investigation[5] has shown that the effect of small alkyl substituents on potency varies (decreasing in the direction of arrows) according to the nature of the central onium atom as follows.



Alkyltrimethylammonium compounds[1,4] fall into the same order as the tetra-alkylammonium salts, ethyltrimethylammonium being the least active, and butyl-, hexyl- and octyl-trimethylammonium compounds equipotent with tetramethylammonium. Maximum potency on the cat anterior tibialis muscle[6] is also reached in this series with BuN^+Me_3, but activity rapidly falls off with larger alkyl groups (Table I).

The studies of Ariëns and his collaborators[7–9] on avian muscle and on the basis of cumulative concentration-response curves obtained on the frog

rectus abdominis muscle show that increase of alkyl chain length also brings a gradual change from a depolarising neuromuscular block to one which is not competitive. Külz[1] records a steady increase from $n = 2$ to $n = 8$ in the activity of the alkyltriethylammonium compounds $Et_3N^+C_nH_{2n+1}$ on the frog gastrocnemius mucle. Rossum and Ariëns[9], however, report a gradual change from competititive to non-competitive action with increasing alkyl chain length, and note the positive correlation of non-competitive affinity, surface activity and fat-solubility.

TABLE I

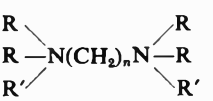
RELATIVE CURARISING ACTIVITY OF ALKYLTRIMETHYLAMMONIUM COMPOUNDS ($R\overset{+}{N}Me_3$) ON CAT

Alkyl group	Relative activity*	Alkyl group	Relative activity*
C_3H_7-	0·8	$CH_3(CH_2)_9-$	0·3
$CH_3(CH_2)_8-$	10·0	$CH_3(CH_2)_{11}-$	0·15
$CH_3(CH_2)_6-$	5·0	$CH_3(CH_2)_{13}-$	0·05
$CH_3(CH_2)_7-$	0·3	$CH_3(CH_2)_{15}-$	0·0

* The activity of $Bu\overset{+}{N}Me_3$ (10) is taken as a standard for comparison.

Ing and Wright[5] have also shown that *N*-methyl-1,2,3,4-tetrahydroquinoline methiodide is more active than the corresponding ethiodide, but that this trend is reversed when the nitrogen is part of a hetero-aromatic system. Thus, pyridine and quinoline ethiodides are more potent than the corresponding methiodides, but strychnine, morphine and nicotine ethiodides, in which the nitrogen-containing ring is saturated, have weaker curariform properties than their methiodides[5,10].

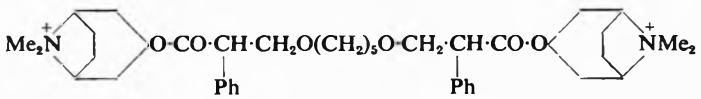
In the polymethylene bistrimethyl- and polymethylene bistriethylammonium series variations in the relative potencies with alkyl group size are evident. The relative order of activity is also dependent on the test preparations. In general, methonium compounds are more potent than their ethonium analogues both on the rat phrenic nerve-diaphragm preparation and in rabbit head drop tests[11]. Thus Barlow, Roberts and Reid[12] have shown that decamethylenebis(dimethylethylammonium)iodide (I; $n = 10$; R = Me; R′ = Et) has about 1/8th of the activity of decamethonium (I; $n = 10$; R = R′ = Me) on the rat phrenic nerve-diaphragm and about 1/15th on the quadriceps of the spinal rabbit. Further substitution of methyl by ethyl groups does not affect the potency on rat diaphragm preparations, but increases it on the rabbit quadriceps upon which decamethylenebis(triethylammonium) (I; $n = 10$; R = R′ = Et) has about 1/4th of the activity of decamethonium (C 10).



(I)

Thesleff and Unna[13] have shown, however, that decaethonium has weak tubocurarine(TC)-like activity, so that replacement of *N*-methyl by *N*-ethyl substituents not only reduces activity, but changes its character. In this respect, the decamethylenebis (alkyldimethylammonium) salts

(I, R = Me, R′ = alkyl) of Rossum and Ariëns[9] are of special interest. Intrinsic activity falls with increasing alkyl chain length, and the depolarising action of C 10 changes through decamethylenebis (dimethylpropyl ammonium) (I, R = Me; R′ = Pr) which shows mixed competitive and depolarising actions, to compounds with extended alkyl chains such as decamethylenebis(dimethylheptylammonium) (I, R = Me, R′ = $CH_3(CH_2)_6$—) which are almost pure non-competitive antagonists. The different modes of action of TC and decamethonium on the cat, chick, frog, in man and in certain other species are well established, but whether this is due to the incorporation of the terminal onium groups of the former into relatively large heterocyclic nuclei or is dependent more upon the presence of additional ring substituents or other factors is not clear. The bisatropinium compounds (II) and (III)[14,15] appear however to exhibit TC-like rather than C 10-like activity, as



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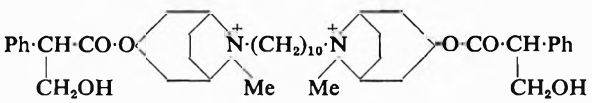
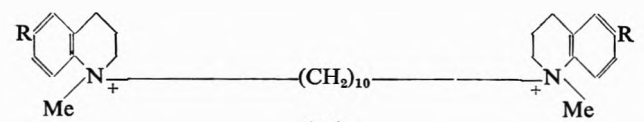


(III)

measured by the production of flaccid paralysis in chicks in compounds (III) and (VIII)[16]. Their potencies are 0·5 and 2·0 respectively compared with TC (1·0) by the rabbit head drop method. Similarly the decamethylenebis-1,2,3,4-tetrahydroquinolinium methiodides (IV; R = H)[17] and (IV; R = OMe)[17], and the decamethylenebis-1,2,3,4-tetrahydroisoquinolinium methiodides (V; R = R′ = H)[18], (V; R = OMe; R′ = H)[18] and (V; R = H, R′ = 3,4-dimethoxybenzyl; laudexium)[19] are antagonised by neostigmine and therefore appear to be TC-like in type. The relative species sensitivities to laudexium which are in the order rabbit > cat > man > mouse > rat suggest that it possesses some resemblance to



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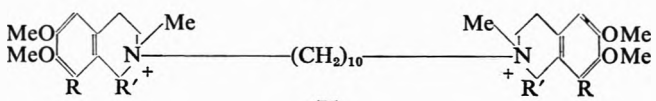

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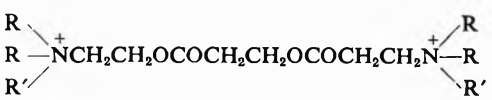

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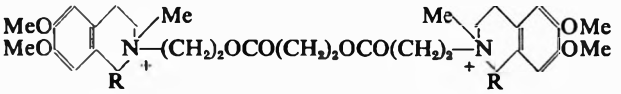

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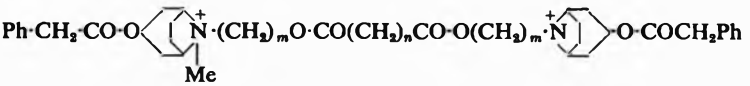


(VIII)

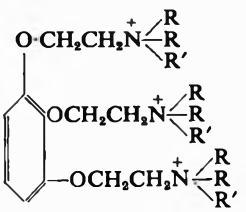
C 10. The depolarising action of suxamethonium (VI; R = R′ = Me) is similarly modified on replacement of the methonium groups by tetrahydroisoquinolinium (VII; R = 3,4-dimethoxybenzyl)[20] and suitably substituted tropinium (VIII; m = 2–3; n = 1–6) groups[16]. Successive replacement of *N*-methyl by *N*-ethyl substituents in suxamethonium lowers the potency as measured in rabbit head drop experiments (Table II)[21], though there is no evidence reported of any change in the type of action.

TABLE II

POTENCY OF SUXAMETHONIUM ANALOGUES (VI)[21]

R	R′	Rabbit head drop dose mg./kg.
Me	Me	0·2
Me	Et	0·8
Et	Me	20·0
Et	Et	12·0

Successive replacement of *N*-ethyl by *N*-methyl groups in the tris-onium compound gallamine (IX; R = R′ = Et) leads to a decrease in curariform potency, though this becomes significant only in the dimethylethylaminoethoxy compound (IX; R = Me; R′ = Et) and the trimethylaminoethoxy compound (IX; R = R′ = Me). There is, however, no departure from the typical TC-like activity of gallamine[23]. In part I of this series[24] we compared the neuromuscular blocking activity of the *NSN*-trisethonium compound, dihexasulphonium tri-ethiodide (X; R = R′ = Et) with that of its methonium analogue (X; R = R′ = Me). The former showed a TC-like neuromuscular blocking action on the cat gastrocnemius muscle, the frog rectus abdominis muscle, the rat diaphragm preparation, in the chick and in rabbit head drop experiments. The latter although qualitatively similar was more recently found to be much less potent (Zoha



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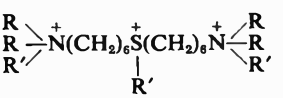
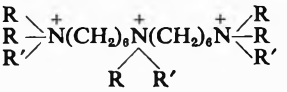


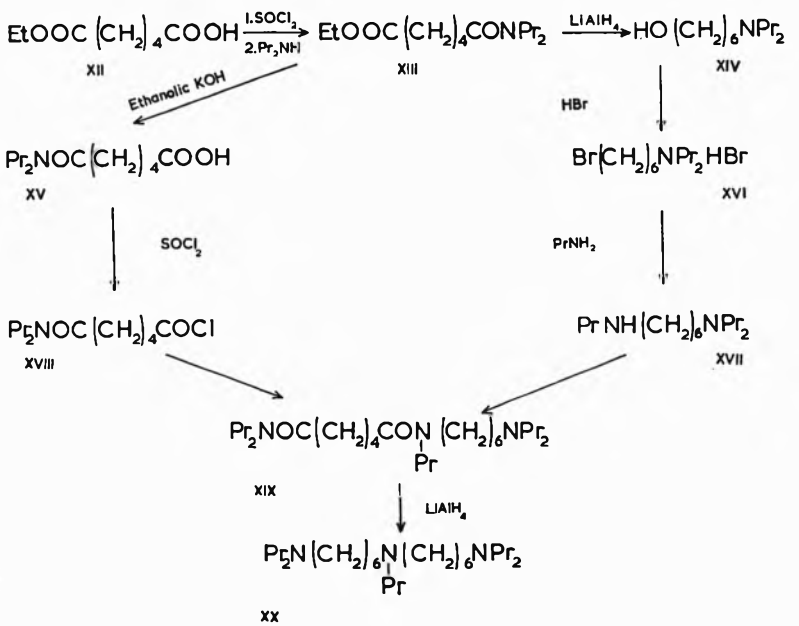
(X)



(XI)

unpublished observations). We have now prepared and examined the related *NSN*-tris-onium compounds described in Table III in order to study the influence of alkyl substituents upon potency and mode of neuromuscular blocking action in this series.

The quaternary compounds 7-ethyl-7-thioniatridecylenebis (dimethylethylammonium) tri-iodide (X; R = Me, R′ = Et), 7-*n*-butyl-7-thioniatridecylenebis (dimethyl-*n*-butylammonium) tri-iodide (X; R = Me, R′ = *n*Bu), 7-methyl-7-thioniatridecylenebis (diethylmethylammonium) tri-iodide (X; R = Et, R′ = Me), and 7-*n*-propyl-7-thioniatridecylenebis (diethyl-*n*-propylammonium) tri-iodide (X; R = Et, R′ = *n*-Pr) were prepared by alkylation of bis-6-dimethylaminohexyl sulphide[24] and bis-6-diethylaminohexyl sulphide[25] as appropriate. In the preparation of *NNN*-tris-onium compounds, an alternative method has been devised for the synthesis of bis-6-diethylaminohexyl ethylamine in improved



yields. This is illustrated by the scheme outlined above for the preparation of the analagous bis-6-di-*n*-propylaminohexyl-*n*-propylamine (XX).

Ethyl *NN*-di-*n*-propyladipamate (XIII), obtained from ethyl hydrogen adipate (XII) by the reactions indicated, was reduced with lithium aluminium hydride to 6-hydroxyhexyldi-*n*-propylamine (XIV), and also hydrolysed to give *NN*-di-*n*-propyladipamic acid (XV). Treatment of

6-hydroxyhexyldi-*n*-propylamine with hydrobromic acid under reflux yielded 6-bromohexyldi-*n*-propylamine hydrobromide (XVI), which with excess *n*-propylamine gave 6-*n*-propylaminohexyldi-*n*-propylamine (XVII). The use of the hydrobromide (XVI) in this reaction rather than the corresponding base[26] prevents cyclisation of the latter and leads to the increased yields.

NN-Di-*n*-propyladipamic acid darkened rapidly when heated with thionyl chloride, but the acid chloride (*NN*-di-*n*-propyladipamoyl chloride, XVIII) was readily obtained by refluxing with thionyl chloride in benzene for exactly seven minutes. Condensation of the product with 6-*n*-propyl-aminohexyldi-*n*-propylamine gave *N*-di-*n*-propylaminohexyl-*NNN*-tri-*n*-propyladipamide (XIV), which on reduction with lithium aluminium hydride yielded bis-6-di-*n*-propylaminohexyl-*n*-propylamine (XX). Quaternisation of the latter with the appropriate alkyl halides gave 7-methyl-7-*n*-propyl-7-azoniatridecylenebis (di-*n*-propylmethylammonium) tri-iodide (XI; R = Pr; R′ = Me), 7-ethyl-7-*n*-propyl-7-azoniatridecylenebis (di-*n*-propylethylammonium) tri-iodide (XI; R = Pr; R′ = Et) and 7,7-di-*n*-propyl-7-azoniatridecylenebis (tri-*n*-propylammonium) tri-iodide (XI; R = R′ = Pr). 7-Methyl-7-ethyl-7-azoniatridecylenebis (diethylmethylammonium) tri-iodide (XI; R = Et; R′ = Me), 7-*n*-propyl-7-ethyl-7-azoniatridecylenebis (diethyl-*n*-propylammonium) tri-iodide (X; R = Et; R′ = Pr) and 7-*n*-butyl-7-ethyl-7-azoniatridecylenebis (diethyl-*n*-butylammonium) tri-iodide (XI; R = Et; R′ = Bu) were obtained from bis-6-diethylaminohexylethylamine[25].

Experimental

Melting points are uncorrected. We are indebted to Miss M. Buchanan for the microanalyses.

NSN-*Tris-onium compounds* (X) were prepared from either bis-6-dimethylaminohexyl sulphide[24] or bis-6-diethylaminohexyl sulphide[25] by refluxing with the appropriate alkyl halide in ethanol, evaporation of the solvent and crystallisation. Reflux times, crystallisation solvents and yields are indicated for each compound in that order, in parentheses.

7-*Ethyl*-7-*thioniatridecylenebis* (*dimethylethylammonium*) *tri-iodide* (35 min.; ethanol; 61 per cent), m.p. 137 to 137·5°. Found: N, 3·6; I, 50·1. $C_{22}H_{51}N_2SI_2$ requires N, 3·7; I, 50·3 per cent.

7-n-*Butyl*-7-*thioniatridecylenebis* (*dimethyl*-n-*butylammonium*) *tri-iodide* (40 min.; ethanol-acetone-ether; 51 per cent), m.p. 131 to 131·5°. Found: N, 3·4; I, 45·35. $C_{28}H_{63}N_2SI_3$ requires N, 3·3; I, 45·3 per cent.

7-*Methyl*-7-*thioniatridecylenebis* (*diethylmethylammonium*) *tri-iodide* (20 min.; ethanol; 94 per cent), m.p. 135 to 136°. Found: N, 3·6; I, 49·6. $C_{23}H_{53}N_2SI_3$ requires N, 3·6; I, 49·4 per cent.

7-n-*Propyl*-7-*thioniatridecylenebis* (*diethyl*-n-*propylammonium*) *tri-iodide* (45 min.; ethanol-ether; 52 per cent), m.p. 125·5 to 126°. Found: N, 3·3; I, 44·3. $C_{29}H_{65}N_2SI_3$ requires N, 3·3; I, 44·5 per cent.

Ethyl NN-*di*-n-*propyladipamate* was prepared from ethyl hydrogen adipate (85 g.) by the method described for the preparation of ethyl *NN*-diethyladipamate[25]. *Ethyl* NN-*di*-n-*propyladipamate* was obtained

as a yellow oil, b.p. 144 to 146°/0·35 mm., n_D^{22} 1·4550 (110 g. 87·6 per cent). Found: N, 5·5. $C_{14}H_{27}O_3N$ requires N, 5·4 per cent.

6-*Hydroxyhexyldi*-n-*propylamine* was prepared from ethyl *NN*-di-*n*-propyladipamate (75·5 g.) by lithium aluminium hydride reduction as described for the preparation of 6-hydroxyhexyldiethylamine[25]. 6-*Hydroxyhexyldi*-n-*propylamine* was obtained as a colourless oil, b.p. 115 to 117°/0·65 mm., n_D^{22} 1·4533 (56·1 g., 95 per cent). Found: equiv. (titration) 200·5; N, 6·9. $C_{12}H_{27}ON$ requires equiv. 201·3; N, 7·0 per cent.

6-n-*Propylaminohexyldi*-n-*propylamine.* 6-Hydroxyhexyldi-*n*-propylamine (55·3 g.) in hydrobromic acid (48 per cent; 30 ml.) was refluxed for 5 hours and evaporated to a thick syrup under reduced pressure. Water (50 ml.) was added and the liquid again evaporated. This procedure was repeated twice after the addition of ethanol (50 ml.). The residual crude 6-bromohexyldi-*n*-propylamine hydrobromide in ethanol (75 ml.) was added slowly (30 min.) to a gently refluxing mixture of *n*-propylamine (100 g.) in ethanol (100 ml.). Refluxing was continued for a further 1½ hours. The excess *n*-propylamine and ethanol were removed by distillation, leaving a solid crystalline mass, which was basified and extracted with ether. Evaporation of the ether and fractionation gave 6-*n*-*propylamino-hexyldi*-n-*propylamine* as a colourless oil, b.p. 115 to 117°/0·45 mm., n_D^{22} 1·4463 (47 g., 70·6 per cent). Found: equiv. (titration) 121·6, N, 11·4. $C_{15}H_{34}N_2$ requires equiv. 121·2, N, 11·6 per cent.

NN-*Di*-n-*propyladipamic acid.* Ethyl *NN*-di-*n*-propyladipamate (27·8 g.) was refluxed for 30 min. with a slight excess of ethanolic potassium hydroxide (130 ml. of 0·9363N) and the bulk of the ethanol removed by distillation. The residual liquid in water (40 ml.) was acidified by addition of hydrochloric acid (50 per cent, 100 ml.), extracted with benzene, dried (Na_2SO_4) and the solvent distilled off. NN-*Di*-n-*propyladipamic acid* was obtained as a yellow viscous oil, b.p. 198°/0·5 mm., n_D^{25} 1·4723, (22·75 g., 91·9 per cent). Found: equiv. (titration) 227·5; N, 6·0. $C_{12}H_{23}O_3N$ requires equiv. 229·3; N, 6·1 per cent.

Bis-6-*di*-n-*propylaminohexyl*-n-*propylamine.* *NN*-Di-*n*-propyladipamic acid (11·6 g.) was dissolved in benzene (10 ml.), refluxed with excess thionyl chloride (6 ml.) for 7 min., and the solute evaporated under reduced pressure; benzene (10 ml.) was added and again evaporated. The acid chloride in benzene (30 ml.) was added slowly (15 min.) to a stirred refluxing solution of excess 6-*n*-propylaminohexyldi-*n*-propylamine (22·4 g.) in benzene (100 ml.). The mixture was refluxed for a further 30 min. and then extracted with hydrochloric acid (10 per cent). The acid extract was basified and extracted with ether, dried (Na_2SO_4) and the solvent removed by distillation. Most of the excess 6-*n*-propylaminohexyldi-*n*-propylamine was recovered by distillation, and the crude *N*-di-*n*-propylaminohexyl-*NN'N'*-tri-*n*-propyladipamide reduced by means of lithium aluminium hydride in ether. Fractional distillation of the product gave *bis*-6-*di*-n-*propylamino-hexyl*-n-*propylamine* as a pale yellow oil, b.p. 211°/0·65 mm., n_D^{21} 1·4582 (16·7 g., 77·5 per cent). Found: equiv. (titration) 142·3; N, 9·8. $C_{27}H_{59}N_3$ requires equiv. 141·9; N, 9·9 per cent.

NN-*Diethyladipamic acid* was prepared from ethyl *NN*-diethyladipamate (100 g.) by the method described for the preparation of *NN*-di-*n*-propyladipamic acid.

NN-*Diethyladipamic acid* was obtained as a yellow viscous oil (83·96 g., 95·7 per cent), b.p. 182°/0·5 mm., $n_D^{20·5}$ 1·4733. Found: equiv. (titration) 201·9; N, 6·7 $C_{10}H_{19}O_3N$ requires equiv. 201·3; N, 7·0 per cent.

Bis-6-diethylaminohexylethylamine was prepared from *NN*-diethyladipamic acid (19·79 g.) and excess 6-diethylaminohexylethylamine (27·8 g.) by the method described for the preparation of bis-6-di-*n*-propylaminohexylethylamine. Bis-6-diethylaminohexylethylamine was obtained as pale yellow oil, b.p. 173 to 176°/0·75 mm., n_D^{25} 1·4588 (19·53 g., 55·9 per cent).

NNN-*Tris-onium compounds* (XI) were prepared from either bis-6-di-*n*-propyl-aminohexyl-*n*-propylamine or bis-6-diethylaminohexylethylamine by refluxing with the appropriate alkyl halide in ethanol, evaporation of the solvent and crystallisation. Reflux time, crystallisation solvent and yields are indicated for each compound in that order, in parenthesis.

7-*Methyl*-7-n-*propyl*-7-*azoniatridecylenebis*(*di*-n-*propylmethylammonium*) *tri-iodide* (10 min.; ethanol; 94 per cent), m.p. 239°. Found: N, 4·9; I, 44·7. $C_{30}H_{68}N_3I_3$ requires N, 4·9; I, 44·7 per cent.

7-*Ethyl*-7-n-*propyl*-7-*azoniatridecylenebis*(*di*-n-*propylethylammonium*)*tri-iodide* (35 min., ethanol-acetone-ether; 66 per cent), m.p. 221°. Found: N, 4·7; I, 42·6. $C_{33}H_{74}N_3I_3$ requires N, 4·7; I, 42·6 per cent.

7,7-*Di*-n-*propyl*-7-*azoniatridecylenebis*(*tri*-n-*propylammonium*)*tri-iodide* (45 min., acetone-ether, 12 per cent), m.p. 206 to 207°. Found: N, 4·5; I, 40·3. $C_{36}H_{80}N_3I_3$ requires N, 4·5; I, 40·7 per cent.

7-*Ethyl*-7-*methyl*-7-*azoniatridecylenebis*(*diethylmethylammonium*)*tri-iodide* (5 min., methanol, 88 per cent), m.p. 227·5 to 228·5°. Found: N, 5·3; I, 48·6. $C_{25}H_{58}N_3I_3$ requires N, 5·4; I, 48·7 per cent.

7-*Ethyl*-7-n-*propyl*-7-*azoniatridecylenebis*(*diethyl*-n-*propylammonium*)*tri-iodide* (30 min., ethanol-acetone-ether, 43 per cent), m.p. 220°. Found: N, 4·9; I, 43·7. $C_{31}H_{70}N_3I_3$ requires N, 4·9; I, 44·0 per cent.

7-*Ethyl*-7-n-*butyl*-7-*azoniatridecylenebis*(*diethyl*-n-*butylammonium*)*tri-iodide* (45 min., acetone-ether, 61 per cent), m.p. 178°. Found: N, 4·6; I, 42·0. $C_{34}H_{73}N_3I_3$ requires N, 4·6; I, 42·0 per cent.

Pharmacology

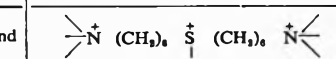
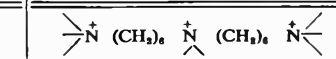
Methods and Results

The methods and materials used have been described elsewhere[24,26]

Neuromuscular blocking activity. To investigate neuromuscular blocking potency in the cat gastrocnemius muscle-sciatic nerve preparation, drugs were administered into the cannulated external jugular vein. None of the compounds tested caused muscular fasciculation or twitching or induced an initial increase in twitch height. XIE was the least potent on this preparation and doses of 0·2 to 0·8 mg./kg. caused a reduction in twitch amplitude, a dose of 0·6 mg./kg. being adequate to reduce it by about 50 per cent. XIC was the most potent member of the series; doses of 0·05 to 0·2 mg./kg. reduced twitch amplitude and 0·1 mg./kg. was

TABLE III

THE INFLUENCE OF CHANGES IN ALKYL GROUP SIZE UPON NEUROMUSCULAR BLOCKING ACTIVITY IN THE CAT, RABBIT, MOUSE, AND FROG

Compound				Cat	Rabbit	Mouse	Frog
				TC = 100	TC = 100	TC = 100	TC = 100
XA	Me_3	Me	Me_3	20	14	5	—
XB	Me_3Et	Et	Me_3Et	26	27	24	14
XC	Me_3Bu	Bu	Me_3Bu	44	33	24	10
XD	$MeEt_3$	Me	$MeEt_3$	87	46	42	16
XE	Et_3	Et	Et_3	95	30	25	25
XF	Et_3Pr	Pr	Et_3Pr	46	51	54	16
							
XIA	Et_3Me	EtMe	Et_3Me	50	52	16	29
XIB	Et_3Pr	EtPr	Et_3Pr	88	69	48	16
XIC	Et_3Bu	EtBu	Et_3Bu	120	155	63	47
XID	Et_3	EtEt	Et_3	100	21	17	50
XIE	$MePr_3$	MePr	$MePr_3$	15	18	11	5
XIF	$EtPr_3$	EtPr	$EtPr_3$	31	46	17	15
XIG	Pr_3	PrPr	Pr_3	20	21	3	13

Pr = *n*-propyl. Bu = *n*-butyl

TABLE IV

THE DURATION OF EFFECT IN THE CAT, AVERAGE DOSES REQUIRED TO PRODUCE RESPIRATORY PARALYSIS IN THE CAT AND PARALYSIS IN THE CHICK IN COMPOUNDS XA TO XF AND XIA TO XIG

Compound	Mean respiratory paralysing dose (mg./kg.)	Respiratory paralysing potency (TC = 100)	Chich paralysis		Paralysing potency (cat) (TC=100)	Duration of effect (cat) in min.
			Dose (mg./kg.)	Potency (chick) (TC=100)		
XA	>2·28	<20	33	50	20	15–25
XB	1·43	30	16	100	26	20–40
XC	1·05	41	20	80	44	10–35
XD	0·70	61	14	115	87	15–45
XE	1·07	45	—	—	95	20–30
XF	0·76	57	19	29	46	30–45
XIA	0·76	57	22	73	50	20–30
XIB	0·48	90	16	100	88	20–30
XIC	0·32	135	12	133	120	20–30
XID	0·85	56	—	—	100	20–30
XIE	>2·82	<20	19	84	15	20–30
XIF	0·81	53	19	84	31	20–30
XIG	1·93	23	33	50	20	15–30

sufficient to cause a 50 per cent reduction. The approximate potencies of these and the other compounds tested are shown in Tables III and IV. The least potent compounds are XB, XIG, XIF and XIE. XD, XIC and XIB are the most potent and XC, XF, XIA occupy intermediate positions. These figures are compared with those of Edwards and others, for XA (1/5 as potent as TC) and XE and XID (equipotent with TC)[26]. The duration of effect varied not only with the dose but with the animal. The ranges of duration of activity (min.) in the cat for an approximate 50 per cent reduction in initial twitch amplitude were as follows; TC,

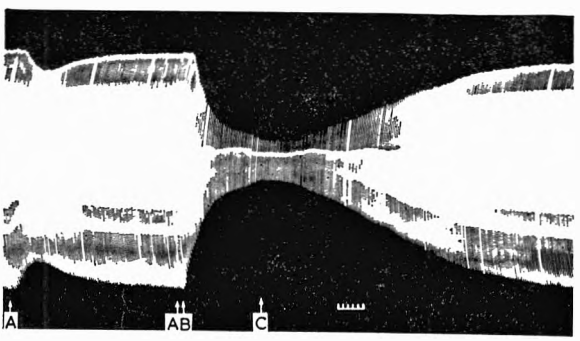


FIG. 1. Ether-potentiation of the effects of XI C on the gastrocnemius muscle-sciatic nerve preparation of the cat.
At A. 0·025 mg./kg. XI C into the external jugular vein.
At B. Ether administration commenced.
At C. Ether administration ceased.
Time = 30 seconds.

15 to 30; XD, 15 to 45; XB, 20 to 40; XC, 10 to 35; XF, 30 to 45; XIA, 20 to 30; XIB, 20 to 30; XIC, 20 to 30; XIG, 15 to 30; XIF, 20 to 30; XIE, 20 to 30. By comparison XA lasted for 15 to 25; XE, 20 to 30 and XID, 20 to 30.

In virtually all respects these compounds behaved like TC. There appears to be no marked qualitative differences from one another or from TC but after XB an indirect tetanus was fairly well maintained. Block was rapidly reversed by edrophonium and more slowly by neostigmine. Potentiation by ether was seen in all; this effect was most striking when XIC was used (Fig. 1).

Rabbit head drop. Comparisons of potency were made with tubocurarine and the experiments were repeated in rabbits which had been given neostigmine (0·1 mg./kg.) by subcutaneous injection 15 minutes earlier. The results are shown in columns 1 and 2 of Table V. Column 3 shows the ratio of the two head drop doses. For all, save compound XF, the ratio is greater than unity indicating a TC-like mode of action.

Acute toxicity in mice. Using 5 or 6 dose levels for each drug and giving each dose to a group of 5 albino mice (average wt. 36 g.) an approximate median lethal dose was estimated. The results are summarised in Table VI. There is fairly good agreement between the toxicity to mice and the paralysing potency estimated on the rabbit and cat.

Paralysing activity in mice. Mice were given an intraperitoneal injection of the drug and placed upon the upper part of a fine wire mesh screen inclined at an angle of 50° to the horizontal. Groups of 5 albino mice (average wt. 36 g.) were used at each dose and 5 or 6 different doses used for each compound. The number of mice in each group which were unable to maintain their position on the screen at each dose was

TABLE V

THE HEAD DROP DOSES (H.D.D.) IN RABBITS OF COMPOUNDS XA TO XF AND XIA TO XIG, AND THE EFFECT UPON THESE OF PRE-TREATMENT WITH NEOSTIGMINE (0·1 mg./kg.)

	1	2	3	
Compound	H.D.D. ± S.E.	H.D.D. ± S.E. after neostigmine	Ratio 2/1	Potency (TC = 100)
XA	2·17 ± 0·059	2·76 ± 0·138	1·22 ($P < 0·01$)	14
XB	1·16 ± 0·047	1·62 ± 0·082	1·40 ($P < 0·01$)	27
XC	0·93 ± 0·071	1·25 ± 0·108	1·34 ($P < 0·05$)	33
XD	0·67 ± 0·031	1·00 ± 0·050	1·50 ($P < 0·01$)	46
XE	0·36 ± 0·017	0·59 ± 0·071	1·64 ($P < 0·01$)	30
XF	0·59 ± 0·035	0·59 ± 0·032	1·00	51
XIA	0·60 ± 0·045	1·51 ± 0·058	2·5 ($P < 0·01$)	52
XIB	0·46 ± 0·024	0·86 ± 0·057	1·9 ($P < 0·01$)	69
XIC	0·20 ± 0·012	0·41 ± 0·036	2·1 ($P < 0·01$)	155
XID	0·51 ± 0·043	0·81 ± 0·057	1·56 ($P < 0·01$)	21
XIE	1·70 ± 0·044	2·98 ± 0·405	1·73 ($P < 0·01$)	18
XIF	0·75 ± 0·052	1·41 ± 0·111	1·36 ($P < 0·01$)	46
XIG	1·47 ± 0·103	2·81 ± 0·179	1·90 ($P < 0·01$)	21

counted after a 30-minute observation period, and from this an approximate 50 per cent paralysing dose (PD 50) was calculated. The results obtained are summarised in Table VI. Once again there is fair agreement between the relative potencies as estimated in mouse, rabbit and cat.

Effects upon blood pressure and respiration in the cat. No depressor effect was observed on the blood pressure. To estimate the average dose required to paralyse respiration, a solution containing 0·1 mg./kg./ml. was infused at a rate of 0·75 ml./min. into the external jugular vein of pentobarbitone-anaesthetised cats using a Palmer's slow injection apparatus. The approximate respiratory paralysing doses are shown in Table IV.

Ganglion blocking activity. Ganglion blocking activity was investigated by noting the effects of up to 2 mg./kg. intravenously of the drug upon the response of the nictitating membrane of the pentobarbitone-anaesthetised cat, to stimulation of the preganglionic fibres of the cervical sympathetic. Only XD (2 mg./kg.) caused any depression of the response but this was much less than that caused by an equal dose of TC.

Chick paralysis. In each case 5 mg./kg. doses were given intraperitoneally to groups of five two-day old chicks and the dose repeated at 30 second intervals until paralysis was seen. With all compounds, a

flaccid TC-like paralysis was observed. The average paralysing doses for each drug are shown in Table IV. When tested on chicks XA caused paralysis which was spastic at first, then becoming flaccid. Dihexasulphonium and dihexazonium have been found to cause a flaccid paralysis (Zoha, unpublished observations).

Frog rectus abdominis muscle. The muscle was set up in a 6 ml. bath containing oxygenated frog Ringer's solution at room temperature. At the doses used (up to 10 μg./ml.) no direct stimulant actions were observed

TABLE VI

THE APPROXIMATE MEDIAN LETHAL DOSE AND PD 50 IN MICE OF COMPOUNDS XA TO XF AND XIA TO XIG

Compound	Approximate median lethal dose (mg./kg.)	PD 50 (mg./kg.)	Potency TC = 100
XA	11·4	7·5	5
XB	4·6	1·6	24
XC	3·5	1·6	24
XD	1·8	0·9	42
XE	1·2	0·8	25
XF	2·6	0·7	54
XIA	2·6	2·4	16
XIB	1·9	0·8	48
XIC	1·4	0·6	63
XID	2·3	1·2	17
XIE	12·7	3·4	11
XIF	5·8	2·2	17
XIG	20	15	3

with any of the compounds tested. All of the compounds (2 to 10 μg./ml.) antagonised contractions produced by acetylcholine (0·3 to 1·0 μg./ml.). The potencies in terms of TC are shown in Table III. XIC was about half as potent as TC but the others showed from 1/20th to 1/4th of the potency.

DISCUSSION

Our findings appear to confirm the observations of Thesleff and Unna[13] and Ariëns and de Groot[22] in the decamethonium series that larger alkyl onium-group substituents favour TC-like activity. Thus compounds XA and XB, in which the terminal onium groups are $Me_3\overset{+}{N}-$ and $Me_2Et\overset{+}{N}-$ respectively show some evidence of decamethonium-like properties, but the remaining members of the present series show purely TC-like activity. (Table VII).

The increase in potency on successive replacement of Me by Et (compounds X A,B,D,E and XI A,D; Table III) in the onium groups of both dihexasulphonium and dihexazonium parallels the influence of similar replacements in the gallamine series (IX)[23], and is in contrast to the decrease in potency which has been observed in the decamethonium[12]

TABLE VII

QUALITATIVE PROPERTIES OF COMPOUNDS XA TO XF AND XIA TO XIG ON THE CAT, CHICK AND FROG RECTUS ABDOMINIS MUSCLE

Compound	Effect on block of:—					Effect of block upon tetanus	Chick paralysis	Effect on frog rectus abdom inis	Effect upon nictitating membrane	Effect upon cat blood pressure
	Neo-stigmine	Edro-phonium	Ether	Adrenaline	Tetanus					
XA	A	A	Prolongs	Transient antagonism	Transient decurarisa-tion	Contraction not sustained	Spastic then flaccid	No direct action. Inhibits ACH con-tractions	0	0
XB	A	A	do.	do.	do.	Contraction fairly well sustained	Flaccid	do.	0	Slight rise
XC	A	A	do.	do.	do.	Contraction not sustained	do.	do.	0	do.
XD	A	A	do.	do.	do.	do.	do.	do.	Slight block	0
XE	A	A	do.	do.	do.	do.	do.	do.	0	Slight rise
XF	A	A	do.	do.	do.	do.	do.	do.	0	do.
XIA	A	A	do.	do.	do.	do.	do.	do.	0	do.
XIB	A	A	do.	do.	do.	do.	do.	do.	0	do.
XIC	A	A	do.	do.	do.	do.	do.	do.	0	do.
XID	A	A	do.	do.	do.	do.	do.	do.	0	0
XIE	A	A	do.	do.	do.	do.	do.	do.	0	Slight rise
XIF	A	A	do.	do.	do.	do.	do.	do.	0	do.
XIG	A	A	do.	do.	do.	do.	do.	do.	0	do.

A = antagonism.
0 = no effect.

and suxamethonium series[21,22]. Thesleff and Unna[13] have also shown in mice and in the chicken sciatic nerve-gastrocnemius muscle preparation that potency increases when Me is replaced by Et in both pentamethonium (XII, $n = 5$, R = Me) and hexamethonium (XII $n = 6$, R = Me),

$$R_3\overset{+}{N}\cdot(CH_2)_n\cdot\overset{+}{N}R_3$$

(XII)

although the actual potencies of compounds of this type are low. No change, however, occurs in the type of action which remains purely TC-like.

It is evident, therefore, that the influence of *N*- or *S*-alkyl substituents cannot be divorced entirely from considerations of inter-onium group spacing. This is shown more clearly by comparison of molecular models of gallamine (IX), hexamethonium, dihexasulphonium and dihexazonium, which reveal almost identical interonium group distances (approximately 9Å; Fig. 2A and B) in all four substances. In reaching this conclusion, it is assumed that no unusual folding of these flexible molecules occurs at the instant of binding at the receptor site, and that the staggered orientation of the positively charged ethoxytriethylammonium groups in gallamine (Fig. 2A) represents their most probable distribution as a result of the natural repulsion of like charges. The structure of tubocurarine (XIII), however, is such that although the molecule cannot be regarded as completely rigid, the spacing of the nitrogen atoms is fixed by considerations of restricted rotation. Moreover, whilst the exact conformation cannot be predicted from models, there is considerable folding of the molecule, which results in reduction of the inter-onium group distance as compared with other estimates of 13 to 15Å based (*a*) on models[14] (*b*) on the number of chain units which separate them (9 carbon and 1 oxygen[11]) and (*c*) on comparison with decamethonium[27]. It is difficult to reconcile the estimate that the terminal groups in decamethonium are only 10Å apart[27] with present views that the hydrocarbon chain is fully extended, giving an *NN* distance of about 15Å. Measurements of interonium group distance on models of tubocurarine suggest that it is probably about 9 to 10 Å, so that in this respect the molecule approximates to the structures of hexamethonium (XII, $n = 6$, R = Me), gallamine (IX), dihexasulphonium (X) and dihexazonium (XI). The hexamethonium-like ganglion block caused by tubocurarine could conceivably be explained on the same basis, but the lack of ganglion-blocking activity in (X) (XI) presents an obvious difficulty.

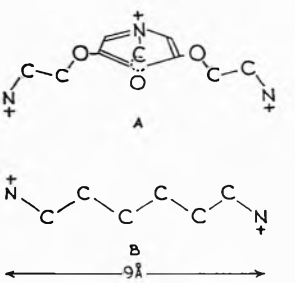


FIG. 2.

Considering now the replacement of Et by larger alkyl substituents in dihexasulphonium and dihexazonium, we have observed that the introduction of a single *n*-propyl group at each quaternary centre as in XF and

XIB leads to a reduction in potency. Potency falls again when a second *n*-propyl substituent is introduced (XIF) and still further when all Et groups have been replaced by *n*-propyl (XIG). Introduction of a single *n*-butyl substituent at each quaternary centre on the contrary enhances potency. These results can be explained in terms of competitive reaction at a receptor surface which presents a repeating pattern of appropriately spaced (9Å) anionic centres, each anionic receptor being in turn associated

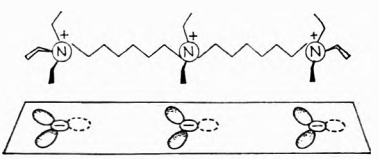


FIG. 3.

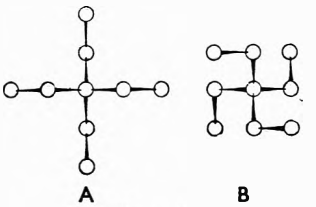


FIG. 4.

with a small number (two or three) of non-ionic satellite receptors at the neuromuscular synapse which ideally are complementary in size and shape to the ethyl substituents (Fig. 3).

Wait and Powell[28] have established the conformation of the tetraethylammonium ion to be that which in projection forms a nordic cross (Fig. 4B), which is in contrast to that of the tetra-*n*-propylammonium (Fig. 4A)[29]. On the assumption then that the swastika-like conformation is [illegible] the alkyltriethylammonium groups of dihexasulphonium and

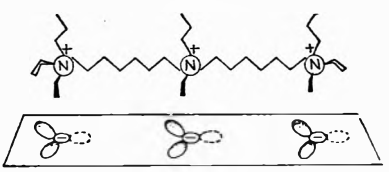


FIG. 5.

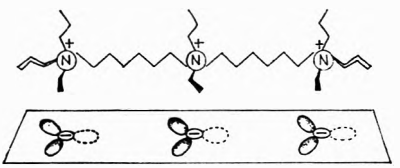


FIG. 6.

dihexazonium, considerable shielding of the charge on the nitrogen atom can be predicted. The competitive nature of the block shown by these compounds, as evidenced for example by reversibility with edrophonium and neostigmine, would probably be fostered by such shielding, which prevents irreversible binding at the receptor surface. The absence of this effect in compounds XA and XB in which Et groups have been replaced by Me groups would permit closer approach to and hence firmer binding with receptors thereby explaining the appearance of decamethonium-like properties which we have observed with these two compounds.

The reduction of potency when a single *n*-propyl substituent is introduced at each quaternary centre in compounds XF and XIB results not only from the partial impairment of fit with satellite receptors at the centre onium group (Fig. 5) but also from the restriction imposed by the *n*-propyl groups on the probability of the molecule reaching a correct orientation with the receptor surface.

The introduction of additional *n*-propyl substituents in compounds XIF and XIG would lead not only to a further impairment of fit with satellite receptors (Fig. 6), but also restrict approach to the receptor surface.

The apparent anomaly of increased activity in the *n*-butyl substituted compound XIC parallels observations on simple monoquaternary compounds[4]. It can be explained in terms of the present hypothesis on the assumption that the surface activity of the *n*-butyl substituents leads to

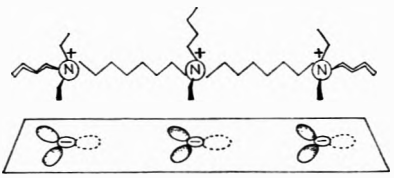


FIG. 7.

the possibility of their alignment as shown in Figure 7, and this more than compensates for any reduction in the probability of the molecule reaching its ideal orientation at the receptor surface.

The hypothesis can be tested further by comparison of equivalent compounds in the dihexasulphonium and dihexazonium series. Replacement of quaternary nitrogen by tertiary sulphur will reduce the goodness of fit with both anionic and satellite receptors by virtue of the increased ionic radius of sulphur (1·04Å) as compared with nitrogen (0·7Å). This acts in several ways (Fig. 8), (*a*) by fractionally increasing interonium

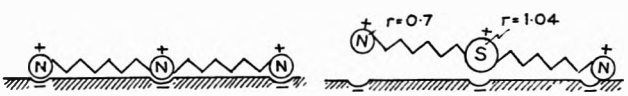


FIG. 8.

group chain length, (*b*) by decreasing the charge density on the central onium group, hence reducing its capacity to bind ionically and (*c*) increasing onium group-receptor distance for one of the two quaternary nitrogens in the molecule. Both (*a*) and (*c*), however, could be negated by limited bending of the hydrocarbon chain. The reduction from two to one in the number of alkyl substituents available on the central onium group for combination with satellite receptors could also contribute to a fall in potency.

Examination of our results reveals only slight reduction of potency when dihexasulphonium triethiodide is compared with dihexazonium triethiodide on the cat, which is of doubtful significance. In man, however, dihexazonium is significantly more potent (Levy, personal communication). Comparison of the azonium compound XIB with its sulphonium analogue also reveals that the latter is significantly less potent in cat, rabbit and mouse.

References

1. Külz, *Pflüg. Arch., ges. Physiol.*, 1922, **195**, 623.
2. Marshall, *Trans. Roy. Soc. Edinb.*, 1914, **50**, 481.
3. Peiser, *Pharmakologische Untersuchungen uber der Wirkung von Tetra-methyl, -äthyl und -propylammonium salzen*, Dissertation, Konigsberg, 1920 (quoted by Ing and Wright[5]).
4. Ing and Wright, *Proc. Roy. Soc.*, 1931, **109B**, 337.
5. Ing and Wright, *ibid.*, 1933, **114B**, 48.
6. Dallemagne and Philippot, *Arch. int. Pharmacodyn.*, 1951, **87**, 127.
7. Ariëns, Simonis and De Groot, *ibid.*, 1955, **100**, 298.
8. Ariëns, Rossum and Simonis, *Arzneimit.-Forsch.*, 1956, **6**, 611.
9. Rossum and Ariëns, *Arch. int. Pharmacodyn.*, 1959, **118**, 393.
10. Trendelenberg, *Heffters Handb. exp. Pharmak.*, Berlin, 1923, **1**, 564.
11. Barlow and Ing, *Brit. J. Pharmacol.*, 1948, **3**, 298.
12. Barlow, Roberts and Reid, *J. Pharm. Pharmacol.*, 1953, **5**, 35.
13. Thesleff and Unna, *J. Pharmacol.*, 1954, **3**, 99.
14. Kimura, Unna and Pfeiffer, *J. Pharmacol.*, 1949, **95**, 149.
15. Kimura and Unna, *ibid.*, 1950, **98**, 286.
16. Haining, Johnson and Smith, *Nature, Lond.*, 1959, **183**, 542.
17. Collier and Taylor, *ibid.*, 1949, **164**, 491.
18. Taylor and Collier, *ibid.*, 1950, **165**, 602.
19. Taylor and Collier, *ibid.*, 1951, **167**, 692.
20. Collier, Gladych, Macauley and Taylor, *ibid.*, 1958, **182**, 1424.
21. Bovet, Bovet-Nitti, Guarino, Longo and Marotta, *Rend. ist. super. Sanita*, 1949, **12**, 106.
22. Ariëns and De Groot, *Arch. int. Pharmacodyn.*, 1954, **99**, 193.
23. Riker and Wescoe, *Ann. N.Y. Acad. Sci.*, 1951, **54**, 373.
24. Edwards, Lewis, Stenlake and Zoha, *J. Pharm. Pharmacol.*, 1957, **9**, 1004.
25. Edwards and Stenlake, *ibid.*, 1955, **7**, 852.
26. Edwards, Lewis, Stenlake and Zoha, *ibid.*, 1958, **10**, suppl., *106T*.
27. Paton and Zaimis, *Brit. J. Pharmacol.*, 1949, **4**, 381.
28. Wait and Powell, *J. chem. Soc.*, 1958, 1872.
29. Zalkin, *Acta Cryst.*, 1957, **10**, 557.

NEUROMUSCULAR BLOCKING AGENTS

PART V. LINEAR *NNNN*-TETRA-ONIUM, *NNSNN*-PENTA-ONIUM AND *NNNNNN*-HEXA-ONIUM COMPOUNDS

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AND FAY STOTHERS†

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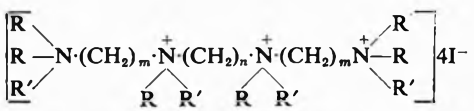
Received May 28, 1959

Preliminary observations upon a series of *NNNN*-tetra-onium compounds (IA to IH), an *NNSNN*-penta-onium compound (II) and an *NNNNNN*-hexa-onium compound (III) have so far indicated that inter-onium distance is more important in determining the type of activity than the overall length of the molecule or the number of onium centres it contains.

IN Part III of this series[1] we made a preliminary report on the neuromuscular blocking activity of the linear *NNNN*-tetra-ethonium compounds, trihexatetrazonium (IA; $m = n = 6$; R = R′ = Et) and tridecatetrazonium (IG; $m = n = 10$; R = R′ = Et), chemically related to the tris-ethonium salts described previously[2,3]. We have now prepared



(I)

the further series of tetra-onium compounds (I), described in Table I, the *NNSNN*-penta-ethonium compound, 7,7,14,21,21-penta-ethyl-7,21-diazonia-14-thioniaheptacosylenebis (triethylammonium) penta-iodide (II), and the *NNNNNN*-hexa-ethonium compound, 7,7,14,14,21,21,28,28-octa-ethyl-7,14,21,28-tetra-azoniatetratriacontylenebis (triethylammonium)

TABLE I

THE CHEMICAL CHARACTERISTICS OF THE LINEAR *NNNN*-TETRA-ONIUM COMPOUNDS, IB TO IH OF THE *NNSNN*-PENTA-ONIUM COMBOUND (II) AND OF THE *NNNNNN*-HEXA-ONIUM COMPOUND (III)

Compound						Melting point (° C.)	Nitrogen (per cent)		Iodine (per cent)	
	m	*n*	R	R′	Formula		Found	Required	Found	Required
IB	6	8	Et	Et	$C_{40}H_{90}N_4I_4$	235–235·5°	4·8	4·9	45·2	44·7
IC	8	6	Et	Et	$C_{42}H_{94}N_4I_4$	246·5–247°	4·8	4·8	43·7	43·6
ID	8	8	Et	Et	$C_{44}H_{98}N_4I_4$	253·5–254°	4·7	4·7	42·5	42·6
IE	6	10	Et	Et	$C_{42}H_{94}N_4I_4$	167·5–168·5°	4·7	4·8	43·2	43·6
IF	10	6	Et	Et	$C_{46}H_{102}N_4I_4$	221–222°	4·7	4·6	41·6	41·6
IH	6	6	Et	*n*-Pr	$C_{48}H_{94}N_4I_4$	197·5–198·5°	4·9	4·8	43·7	43·6
II					$C_{46}H_{103}N_4SI_5$	165·5–166·5°	4·2	4·1	46·2	46·0
III					$C_{58}H_{130}N_6I_6$	248°	5·0	5·0	45·6	45·5

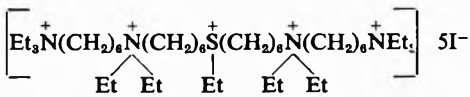
TABLE II

A COMPARISON OF SOME OF THE QUALITATIVE PROPERTIES OF COMPOUNDS IA, IB, IC, ID, IE, IF, IG, IH, II AND III ON THE CAT, CHICK, AND FROG RECTUS ABDOMINIS MUSCLE

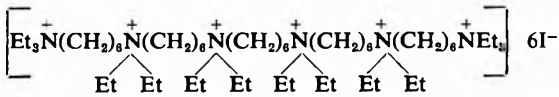
Compound	Effect on block of						Nature of tetanus during block	Effect of tetanus on block	Chick paralysis	Frog rectus	General characteristics
	Neo-stigmine	Edro-phonium	Ether	Adrenaline	TC	C 10					
IA	−	−	+	−	+	−	Poorly held	Decurarisation	Flaccid	No. Stimuln. Antag. Ach	TC-like
IB	−	−	+	±	+	−	,,	,,	,,	,,	,,
IC	−	−	+	±	+	−	,,	,,	,,	,,	,,
ID	±	±	+	±	+	−	,,	,,	,,	,,	,,
IE	±	−	+	±	+	±	,,	Slight decurarisation	,,	,,	,,
IF	0	+	0	±	+	+	Poorly held in most expts	None	Spastic-flaccid	,,	Transitional (?)
IG	0	+			−	+	Fairly well maintained	,,	,,	,,	C 10
IH	−	−	+	±	+	−	Poorly held	,,	Flaccid	,,	TC-like
II	±	±	+	±	+	−	,,	Decurarisation	,,	,,	,,
III	±	−	+	±	+	±	,,	,,	,,	,,	,,

+ = potentiation, addition or prolongation − = antagonism ± = partial antagonism 0 = no effect

hexa-iodide (III). Preliminary pharmacological observations on these compounds are recorded below.



(II)



(III)

The experimental methods and materials have been described in detail elsewhere[2,3]. Table II summarises some of the properties of the new compounds and includes for comparison trihexatetrazonium tetra-ethiodide (IA) and tridecatetrazonium tetra-ethiodide (IG)[1]. Our preliminary observations indicate that the neuromuscular blocking actions of compounds IB, IC, ID, IE, IH, II and III qualitatively resemble those of tubocurarine (TC) rather than those of decamethonium (C 10). These compounds all caused a flaccid paralysis in chicks, had no direct stimulant actions on the isolated frog rectus abdominis muscle and on this tissue antagonised the action of acetylcholine. Most of their other properties were TC-like but we noted that ID, IE, II and III were incompletely antagonised by neostigmine and in addition ID and II incompletely antagonised by edrophonium. These observations may be significant should these compounds be considered for clinical trial in anaesthesia. Of the compounds tested IF was in some ways the most interesting. In some experiments it caused twitching of the limbs, had a very prolonged action and block was intensified by edrophonium and unaffected by neostigmine. Block was not potentiated by ether and in the chick, IF caused an initial spastic paralysis which changed to flaccid. It had no direct stimulant action on the frog rectus abdominis muscle.

Although our observations on the compounds described are incomplete, certain interesting trends are shown. It appears that inter-onium distance is more important in determining the type of activity than the overall length of the molecule and the number of onium centres which it contains. Compounds in which n and $m = 6$ or 8 are essentially TC-like in their qualitative effects but when $n = 6$ and $m = 10$, C 10-like activity appears and is increased when $n = m = 10$.

Acknowledgements. We wish to acknowledge the technical assistance of Miss Gladys Marren.

REFERENCES

1. Edwards, Lewis, Stenlake and Zoha, *J. Pharm. Pharmacol.*, 1958, **10**, Suppl., *122T.*
2. Edwards, Lewis, Stenlake and Zoha, *ibid.*, 1957, **9**, 1004.
3. Edwards, Lewis, Stenlake and Zoha, *ibid.*, 1958, **10**, Suppl., *106T.*

DISCUSSION

After Mrs. Stothers presented the papers there was a DISCUSSION. The following points were made.

The introduction of an *iso*propyl group into the study had been considered but serious interactions might arise as the grouping was bulky. The metabolism of the compounds had not been studied, but the rapidity of action of most of the compounds suggested that this did not occur. The *NSN* compounds were excreted unchanged.

Short Communication

NEUROMUSCULAR BLOCKING ACTIVITY IN SOME *NS*-BIS-ONIUM COMPOUNDS

By T. C. Muir and J. J. Lewis

From the Department of Materia Medica and Therapeutics, University of Glasgow

Received April 20, 1959

Studies by Edwards and his colleagues[1–4] on linear *NNN*, *NSN* and *NNNN*-onium salts have shown that these possess decamethonium (C 10), tubocurarine (TC)-like and transitional properties. *NS*-Compounds with muscle relaxant properties have been investigated by Bovet and his co-workers[5] and Walker[6]. Walker, using the rabbit head drop method, has reported that decamethylene-1,10-bisdimethylsulphonium di-iodide and decamethylene-1-dimethylsulphonium-10-trimethylammonium di-iodide have powerful C 10-like activity but are less potent than C 10 itself.

To gain a more complete picture of the properties of linear *NS*-compounds for comparison with the *NNN*, *NSN* and *NNNN*-derivatives of Edwards and others, we have investigated the properties of octamethylene-1-ethylmethylsulphonium-8-dimethylethylammonium di-iodide (I), decamethylene-1-dimethylsulphonium-10-trimethylammonium di-iodide (II) and octamethylene-1-dimethylsulphonium-8-trimethylammonium di-iodide (III).

The techniques and materials used have been described elsewhere[1–4].

Using 2 to 4 kg. cats anaesthetised by intraperitoneal sodium pentobarbitone the contractions of the gastrocnemius muscle in response to indirect stimulation *via* the scientic nerve were recorded. I (0·1 to 0·5 mg./kg.), II (0·05 to 0·1 mg./kg.) and III (0·1 to 0·5 mg./kg.) reduced twitch amplitude. II (0·025 mg./kg.) caused an initial increase of twitch height without subsequent reduction in its amplitude. When I was used muscular twitching and fasciculation were sometimes observed. I, II and III all resembled C 10 rather than TC; the similarity was closest with II, while III had transitional properties and was additive with both C 10 and TC (Fig. 1). C 10 was approximately 2·5 times more potent than II, ten times more potent than I and eight times more potent than III. After I, II or III the response of the partially blocked muscle to indirect tetanisation was well maintained. Neostigmine (0·05 to 0·1 mg./kg.) and edrophonium (0·5 mg./kg.) either potentiated or had little effect on the intensity of block due to I, II or III. Adrenaline (0·05 mg./kg.) and potassium chloride (15 to 20 mg./kg.) temporarily antagonised the block. Eserine (0·5 to 1·0 mg./kg.) or ether anaesthesia had little effect. The neuromuscular blocking effects of I, II and III were additive with each other and those of C 10 and with the exception of III antagonised the actions of TC.

When tested on the isolated frog rectus abdominis muscle I, II and III (1·0; 0·2; 1·0 mg./ml. respectively) caused a direct contraction and

augmented the stimulant effects of acetylcholine (0·1 μg./ml.). II was the most potent compound of the group. The order of potency was C 10 > II > III > I. When injected intraperitoneally into day-old chicks (10 mg./kg. every 30 seconds until death ensued) II produced a typical

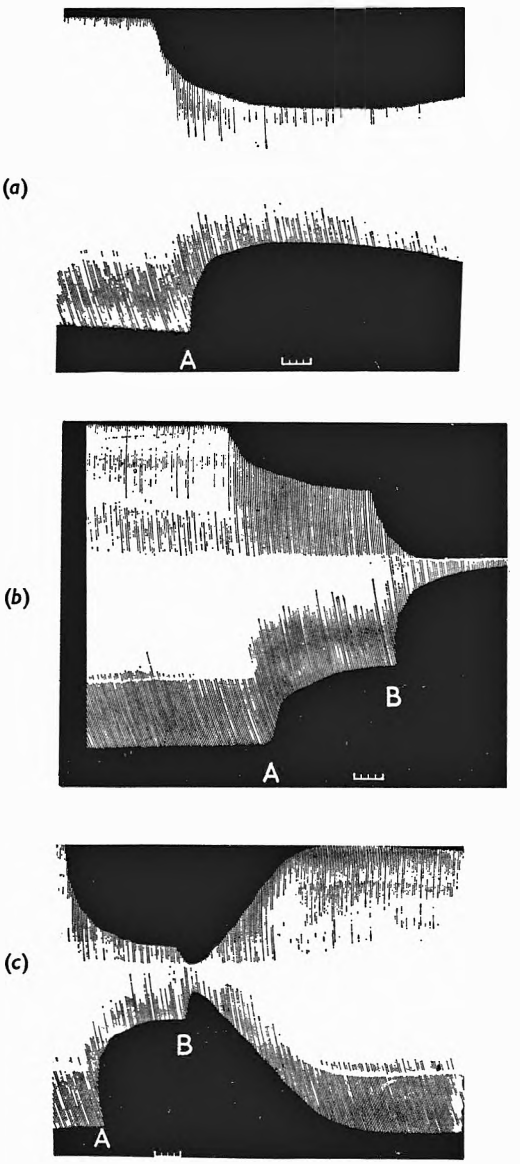


FIG. 1. Cat gastrocnemius-sciatic preparation. Pentobarbitone anaesthesia. Indirect stimulation via sciatic nerve; contraction downwards. Drugs administered intravenously. (*a*) At A, III 0·15 mg./kg., (*b*) at A, III 0·15 mg./kg., at B, C 10, 0·02 mg./kg., (*c*) at A, III 0·15 mg./kg., and at B, TC 0·2 mg./kg.

C 10-like spastic paralysis. I and III, less potent than II, produced an initial spastic paralysis which became flaccid prior to death. The order of potency was C 10 > II > I > III.

I, II and III were injected intraperitoneally into groups of ten mice and all three compounds showed paralysing activity followed by convulsions and death. The effects produced qualitatively resembled those produced by C 10. I had an approximate PD50 of 4·07 mg./kg. and an approximate LD50 of 11·09 mg./kg. The figures for II were 4·1 and 9·33, for III, 3·05 and 24·27, for C 10, 1·78 and 3·81; and TC, 0·13 and 0·26.

In doses sufficient to produce partial neuromuscular block (0·05 to 0·5 mg./kg.) I, II and II had no effect upon the blood pressure level of sodium pentobarbitone-anaesthetised cats, but 250 to 500 μg./kg. of II caused a prolonged rise. The average doses required to paralyse respiration were obtained by infusing the drug solution (I, 1 mg./ml.; II, II and C 10, 0·1 mg./ml.) intravenously at a rate of 0·75 ml./min. into the femoral vein of anaesthetised cats using a Palmer's constant rate infusion apparatus. The values obtained were as follows: I, 1·4 mg./kg.; II, 0·36 mg./kg.; III, 0·43 mg./kg.; C 10, 0·11 mg./kg.

In doses of from 1 to 6 times the muscle relaxant dose I, II or III did not reduce the response of the nictitating membrane of the cat to electrical preganglionic stimulation of the cervical sympathetic but after II (0·3 mg./kg.) there was an increase in amplitude indicating a possible ganglion stimulant action.

Discussion

I, II and III resemble C 10 rather than TC and so differ from dioctasulphonium and dioctazonium which have predominantly TC-like properties and resemble didecasulphonium which is C 10-like[2]. It seems, therefore, that increasing the number of onium centres increases the tendency to TC-like activity. Barlow[7] and Thesleff and Unna[8] have shown that stepwise preplacement of methyl by ethyl in C 10 alters activity from C 10-like to TC-like. Replacement of one quaternary N by tertiary S in C 10 does not alter the type of activity but reduces potency. In compounds I and III activity is mainly C 10-like but potency is very much reduced; both compounds cause a mixed spastic to flaccid paralysis in chicks but unlike I, III is not antagonised being potentiated by TC and C 10. Of these compounds III is the more potent, thus, introduction of an ethyl group lowers potency. Paton and Zaimis[9] have shown that octamethylene-α-ω-bistrimethyl: ammonium chloride has weak C 10-like properties on the cat tibialis preparation. III, in which one quaternary nitrogen has been replaced by tertiary sulphur, also has C 10-like activity. Both I and III have mainly methyl substituents on the onium atoms so that C 10-like activity is not unexpected.

Mr. D. M. Brown tells us that in the straight chain NS bis-ethonium series compounds with polymethylene chains containing 4, 5 or 6 members are potent ganglion blocking agents with little or no neuromuscular blocking activity.

Acknowledgements. We thank Mr. David M. Brown of Beecham Research Laboratories Ltd., for compounds I, II and III, Dr. J. B. Stenlake for help with the chemical nomenclature and Miss Sheila Grace and Mr. Peter Leitch for technical assistance.

REFERENCES

1. Edwards, Lewis, Stenlake and Zoha, *J. Pharm. Pharmacol.*, 1957, **9**, 1004.
2. Edwards, Lewis, Stenlake and Zoha, *ibid.*, 1958, **10**, Suppl., *106T.*
3. Edwards, Lewis, Stenlake and Zoha, *ibid.*, 1958, **10**, Suppl., *122T.*
4. Edwards and Stenlake, *ibid.*, 1955, **7**, 852.
5. Fusco, Palazzo, Chiavarelli and Bovet, *Gazetta*, 1949, **79**, 129.
6. Walker, *J. chem. Soc.*, 1950, **1**, 193.
7. Barlow, Roberts and Reid, *J. Pharm. Pharmacol.*, 1953, **5**, 35.
8. Thesleff and Unna, *J. Pharmacol.*, 1954, **3**, 110.
9. Paton and Zaimis, *Nature, Lond.*, 1948, **161**, 718.

After Mr. Muir presented the communication there was a DISCUSSION.

THE GANGLIONIC BLOCKING ACTIVITY OF A SERIES OF TERTIARY SULPHONIUM QUATERNARY AMMONIUM SALTS

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A number of polymethylene bis-tertiary sulphonium quaternary ammonium salts have been examined for their ganglionic blocking activity on the superior cervical ganglion of the cat. The effect of substituting different alkyl groups on the sulphur and nitrogen atoms and varying the length of the polymethylene chain has been investigated. Maximum activity was found in the tetramethylene di-ethyl sulphonium tri-ethyl ammonium salt, which was about three times as active as hexamethonium. With tri-, penta- and hexamethylene salts maximum activity occurs when there are a total of 13 to 15 carbon atoms in the molecule. It is concluded that the substitution of sulphur for nitrogen in the bis-quaternary ammonium salts does not necessarily lead to a reduction in relative potency. The importance of the groupings on the "onium" centres in producing ganglionic block is stressed.

The effect of substituting sulphur for nitrogen in polymethylene bis-quaternary ammonium salts has been investigated by several workers. Walker[1] has shown that replacing one nitrogen with sulphur in decamethonium led to a decrease in neuromuscular blocking activity. Muir and Lewis[2] confirmed this and demonstrated that the type of neuromuscular block produced by analogous sulphur-nitrogen compounds is unaffected. Barlow and Vane[3] synthesised a number of bis-alkyl tertiary sulphonium analogues of hexamethonium which proved to have less ganglionic blocking activity than their corresponding bis-quaternary ammonium salts. Wien[4] reported the replacement of nitrogen by sulphur in hexamethonium and its homologues to reduce activity. From these investigations it would appear that the replacement of nitrogen by sulphur in polymethylene bis-alkylammonium salts causes a decrease in relative activity.

We have investigated the ganglionic blocking activity of a series of straight chain polymethylene tertiary sulphonium quaternary ammonium salts synthesised by Doyle and Stove[5]. Among the compounds tested a number have been shown to possess ganglionic blocking activity in excess of hexamethonium.

Methods

The ganglionic blocking activity of compounds was assessed on the cat nictitating membrane preparation. The method was slightly modified from that described by Barlow and Vane[3]. Cats were anaesthetised with ether followed by intravenous chloralose-urethane mixture (0·7 per cent chloralose, 2·8 per cent urethane, 8·0 ml./kg.). A square wave stimulus of 1·0 millisecond duration and frequency of 14 shocks per second was

applied for 15 seconds every 3 minutes to the preganglionic fibres of the superior cervical ganglion. The voltage was adjusted to give just maximal contraction of the membrane. The nictitating membrane completely relaxed after each period of stimulation. The compounds were administered intravenously, into the femoral vein, 30 seconds before stimulation. The dose of hexamethonium bromide to give approximately 50 per cent inhibition of contraction (0·5 to 1·0 mg./kg.) was

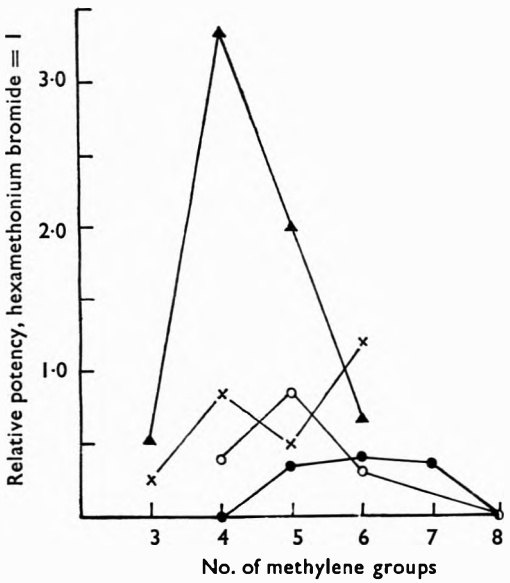

FIG. 1. Relationship between sympathetic ganglion blocking activity and chain length in a series of polymethylene bis-tertiary sulphonium quaternary ammonium salts.

General formula: $R_1R_2S^+(CH_2)_nN^+R_3R_4R_5$

	R_1	R_2	R_3	R_4	R_5
●—●	CH_3	CH_3	CH_3	CH_3	CH_3
○—○	CH_3	C_2H_5	CH_3	CH_3	C_2H_5
×—×	CH_3	C_2H_5	CH_3	C_2H_5	C_2H_5
▲—▲	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5

determined. Three doses of hexamethonium were then bracketed with two doses of the test compound and from the log-dose response lines obtained the relative potency of the compound was calculated.

The acute intravenous toxicities were determined in male mice weighing 18 to 22 g. and expressed as the LD50 in mg./kg.

RESULTS

Figure 1 illustrates the alteration in ganglionic blocking activity as a result of increasing the length of the polymethylene chain and substituting successively ethyl groups for methyl groups on the sulphur and nitrogen atoms. Compounds with methyl groups only on the sulphur and nitrogen have demonstrable ganglionic blocking activity, but this is always less

than hexamethonium. The pentamethylene, hexamethylene and heptamethylene compounds are equipotent, and have a relative ganglionic blocking activity of about 0·4 times hexamethonium. The tetramethylene and octamethylene compounds are inactive. When an ethyl group is substituted for a methyl group on both the sulphur and nitrogen atoms the relative activity within the series increases; the tetramethylene derivative has an activity of 0·4 times hexamethonium, while maximum activity occurs with the pentamethylene derivative which has a potency of 0·85

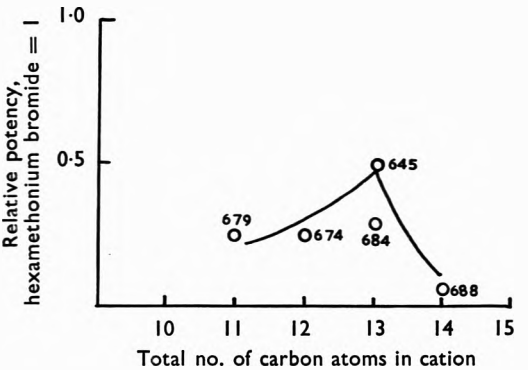

FIG. 2. Relationship between sympathetic ganglion blocking activity and total number of carbon atoms in the cation of a series of trimethylene bis-tertiary sulphonium quaternary ammonium salts.

General formula: $R_1R_2S^+(CH_2)_3N^+R_3R_4R_5$

Code No.	R_1	R_2	R_3	R_4	R_5
679	C_2H_5	CH_3	C_2H_5	C_2H_5	CH_3
645	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5
674	CH_3	CH_3	C_3H_7n	C_3H_7n	CH_3
684	CH_3	C_2H_5	C_3H_7n	C_3H_7n	CH_3
688	CH_3	C_3H_7n	C_3H_7n	C_3H_7n	CH_3

times hexamethonium. As the chain length is increased activity falls away rapidly and no significant ganglionic blocking activity is detectable in the octamethylene salt.

When a further methyl group is replaced by an ethyl group on the nitrogen atom, maximum activity occurs with the hexamethylene compound—the ethylmethylsulphonium di-ethylmethylammonium salt. This compound was slightly more active than hexamethonium. The higher members of this series, however, have not been investigated and therefore a complete picture of the variation in activity within the series has not been obtained.

Replacement of all the methyl groups by ethyl groups markedly alters the activity. There is a sharp increase in activity between the compounds having 3 or 4 carbon atoms in the chain. While the former is only 0·5 times as active as hexamethonium the latter is 3·3 times as active. The pentamethylene derivative is less active than the tetramethylene derivative, but is still twice as active as hexamethonium. The hexamethylene derivative has a relative potency of only 0·6.

TABLE I

SYMPATHETIC GANGLION BLOCKING ACTIVITY AND INTRAVENOUS TOXICITIES OF A SERIES OF $R_1R_2.S^+(CH_2)_4N^+R_3R_4R_5$ SALTS

Code number	Substituent groups					Toxicity in mice. LD_{50} mg./kg. I.V.	Ganglionic block hexamethonium bromide = 1·0
	R_1	R_2	R_3	R_4	R_5		
BRL 443	CH_3	CH_3	CH_3	CH_3	CH_3	194	<0·02
534	CH_3	C_2H_5	CH_3	CH_3	CH_3	170	0·2
519	CH_3	C_2H_5	CH_3	CH_3	C_2H_5	119	0·4
524	CH_3	CH_3	CH_3	C_2H_5	C_2H_5	103	0·4
644	C_2H_5	C_2H_5	CH_3	CH_3	C_2H_5	99	0·9
525	CH_3	C_2H_5	CH_3	C_2H_5	C_2H_5	54	0·9
653	CH_3	C_2H_5	C_2H_5	C_2H_5	C_2H_5	25	1·3
611	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	45	3·3
530	CH_3	CH_3	CH_3	C_3H_7n	C_3H_7n	75	0·6
683	CH_3	CH_3	CH_3	C_3H_7i	C_3H_7i	26	1·8
678	CH_3	C_2H_5	CH_3	C_3H_7i	C_3H_7i	26	2·5
654	CH_3	C_3H_7i	CH_3	C_3H_7i	C_3H_7i	20	0·4
531	CH_3	C_3H_7n	CH_3	C_3H_7n	C_3H_7n	40	0·2
652	CH_3	C_2H_5	C_3H_5	C_3H_7n	C_3H_7n	50	1·4
667	C_2H_5	C_3H_7n	C_2H_5	C_2H_5	C_3H_7n	31	0·4
589	CH_3	CH_3	CH_3	C_4H_9n	C_4H_9n	41	0·5

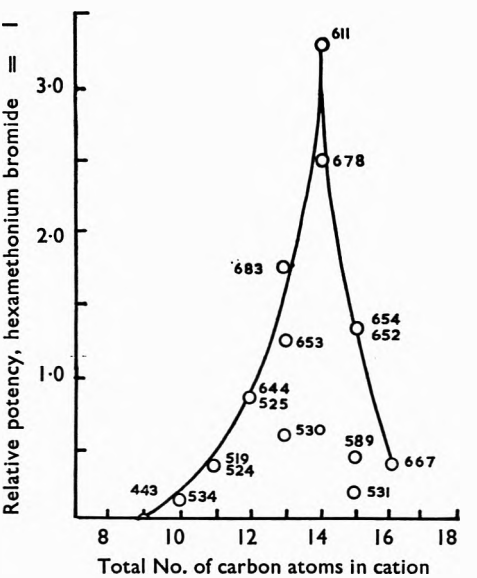

FIG. 3. Relationship between sympathetic ganglion blocking activity and total number of carbon atoms in the cation of a series of tetramethylene bis-tertiary sulphonium quaternary ammonium salts.

Numbers refer to structures shown in Table I.

The effect on the ganglionic blocking activity of substituting in turn methyl groups by larger groups in the tri-, tetra-, penta- and hexamethylene series was investigated further. The variation in activity of the compounds in the trimethylene series is illustrated in Figure 2. Maximum ganglionic blocking activity occurs when only ethyl groups are present on the sulphur and nitrogen atoms. When there are more than a total of 13 carbon atoms in the molecule activity is virtually abolished.

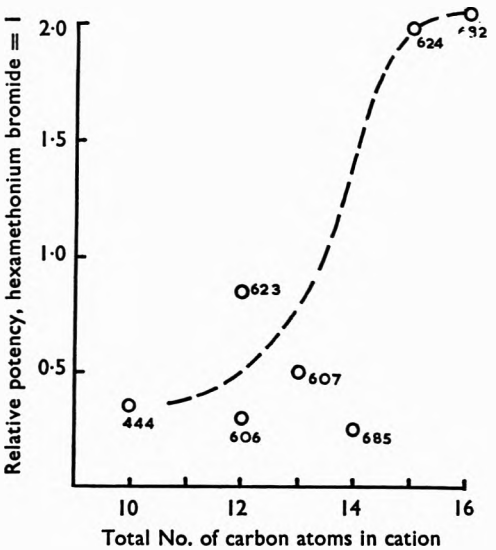

FIG. 4. Relationship between sympathetic ganglion blocking activity and tota number of carbon atoms in the cation of a series of pentamethylene bis-tertiary sulphonium quaternary ammonium salts.

General formula: $R_1R_2S^+(CH_2)_5N^+R_3R_4R_5$

Code No.	R_1	R_2	R_3	R_4	R_5
444	CH_3	CH_3	CH_3	CH_3	CH_3
623	CH_3	C_2H_5	CH_3	CH_3	C_2H_5
606	CH_3	CH_3	CH_3	C_2H_5	C_2H_5
607	CH_3	C_2H_5	CH_3	C_2H_5	C_2H_5
624	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5
685	CH_3	CH_3	CH_3	C_3H_7n	C_3H_7n
682	CH_3	C_2H_5	C_2H_5	C_3H_7n	C_3H_7n

In the tetramethylene series a different picture evolves. The variations in the groupings and their relative ganglionic blocking activities obtained are given in Table I and are illustrated graphically in Figure 3. Maximum activity in this series again occurs when only ethyl groups are present on the sulphur and nitrogen atoms, but in this instance the compound is about 3 times as active as hexamethonium. Larger or smaller groups than ethyl on the sulphur and nitrogen atoms leads to diminished activity. There is also an interesting relationship between the compounds possessing *n*-propyl and isopropyl groups. A comparison between the dimethyl sulphonium di-isopropyl methyl ammonium salt (B.R.L. 683) and the

corresponding *n*-propyl isomer (B.R.L. 530) and a comparison between the methyl isopropyl sulphonium methyl di-isopropyl ammonium salt (B.R.L. 654) and the *n*-propyl isomer (B.R.L. 531) shows that the *n*-propyl compound is in both instances much less active than the corresponding isopropyl isomer.

The compounds prepared with the pentamethylene chain form a rather incomplete picture. The activities are expressed graphically in Figure 4. Maximum activity again occurs when only ethyl groups are present on the sulphur and nitrogen, but the ethylmethylsulphonium di-*n*-propyl ethyl

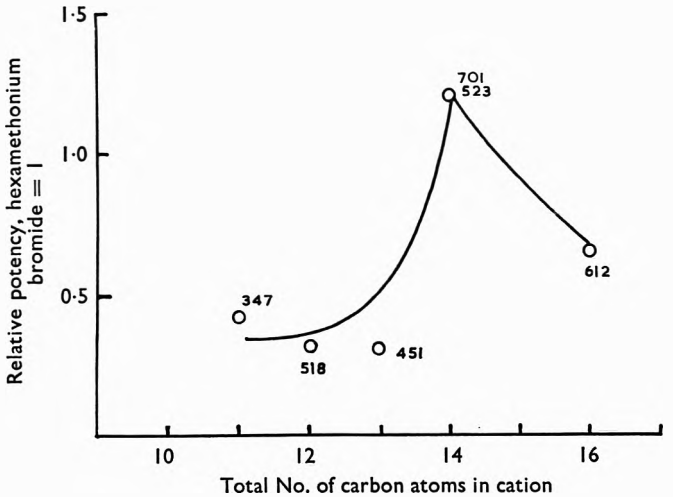

FIG. 5. Relationship between sympathetic ganglion blocking activity and the total number of carbon atoms in the cation of a series of hexamethylene bis-tertiary sulphonium quaternary ammonium salts. General formula: $R_1R_2S^+(CH_2)_6N^+R_3R_4R_5$.

Code No.	R_1	R_2	R_3	R_4	R_5
347	CH_3	CH_3	CH_3	CH_3	CH_3
518	CH_3	C_2H_5	CH_3	CH_3	CH_3
451	CH_3	C_2H_5	CH_3	CH_3	C_2H_5
701	C_2H_5	C_2H_5	CH_3	CH_3	C_2H_5
523	CH_3	C_2H_5	CH_3	C_2H_5	C_2H_5
612	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5

ammonium derivative is of the same order of activity. Compounds with larger groups than ethyl on the sulphur were not prepared, and hence it has been impossible to determine at which stage maximum activity does occur.

The ganglionic blocking activity in the hexamethylene series is illustrated in Figure 5. Maximum activity occurs with the diethyl sulphonium dimethylethyl ammonium salt, and the ethylmethyl sulphonium diethylmethyl ammonium salt. Both these compounds are slightly more active than hexamethonium. The diethyl sulphonium triethyl ammonium salt is about 0·6 times as active as hexamethonium. Compounds possessing larger groups on the sulphur and nitrogen were prepared, but none of these showed any significant ganglionic blocking activity.

Discussion

To achieve the maximum ganglionic blocking activity 4 carbon atoms are required in the carbon chain separating the sulphur and nitrogen atoms and the presence of only ethyl groups on the nitrogen and sulphur atoms is necessary. Barlow and Vane[3] similarly found that maximum activity in the hexamethylene bis-alkyl sulphonium series occurred when only ethyl groups were attached to the sulphur atoms. In the compounds with 3 carbon atoms in the chain the activity is not marked, but when the chain length is increased to 5 carbon atoms compounds with activity of twice that of hexamethonium have been obtained. Increasing the length of the chain to 6 carbon atoms likewise results in compounds with slightly greater activity than hexamethonium, but activity in this series follows a somewhat different pattern than seen in the preceding series. Relative activity would appear to follow more closely the change in activity seen with the bis-nitrogen compounds when the methyl groups are substituted in turn by ethyl groups as shown by Wien, Mason, Edge and Langston[6]. These workers found that the penta- and hexamethylene bis-dimethylethyl quaternary ammonium salts were the most active of the compounds tested, and of the 4 carbon chain compounds the bis-diethylmethyl ammonium salt was the most active. In the sulphur-nitrogen series the most active hexamethylene derivatives are the diethyl sulphonium dimethylethyl ammonium salt and the ethylmethylsulphonium diethylmethyl ammonium salts. Both are equiactive.

Wien and his colleagues also showed that the salt of hexamethylene bis-triethylammonium was practically devoid of ganglionic blocking action, but had a significant neuromuscular paralysing action. No such alteration in effect has been demonstrated in the sulphur-nitrogen series even with the substitution of larger groups. The shorter chain sulphur-nitrogen compounds would seem to be remarkably free from neuromuscular blocking action.

The modifications to structure of mono-quaternary and bis-quaternary ammonium salts which have shown ganglionic blocking activity has been investigated by several workers. Burn and Dale[7] found that substitution of the methyl groups by ethyl groups in tetramethylammonium abolished nicotinic stimulatory action on the blood pressure while maintaining the nicotine paralysing action. Subsequently Paton and Zaimis[8] demonstrated in a series of polymethylene bis-trimethylammonium salts the importance of the distance between the two onium centres. They showed that maximum activity occurred when the polymethylene chain contained 5 or 6 carbon atoms, the hexamethylene derivative being 20 times as active as tetraethylammonium. Wien and others[6] extended this work and investigated the effect of substitution of methyl by ethyl on the nitrogen atoms. They showed that optimal activity depended on both chain length and the nature of the substituents on the nitrogen atoms. The results of the present investigation emphasise the importance of the groupings on the onium centres and also stresses the fact that 5 to 6 carbon atoms separating the onium centres are not necessary for optimal activity. Nevertheless, depending on the type of onium centre present, an optimum

number of carbon atoms would appear to be necessary. In tertiary sulphonium quaternary ammonium compounds optimal activity occurs when there are only 4 carbon atoms in the polymethylene chain. However, to ensure an adequate fit on a receptor site, and so inducing ganglionic block, both the total length and general shape of the molecule appear to be of importance. That the volumes and effective radii of the groupings on the onium centre play a part in determining activity is evident from the difference in activity in the isomeric compounds containing *n*-propyl and *iso*propyl groups. The end groupings probably regulate the approach of the molecule to the receptor site and maximum ganglionic blocking activity is attained when there is correct electrolytic balance between drug and receptor site.

Acknowledgements. Our thanks are due to Mr. F. P. Doyle and Mr. E. R. Stove for synthesising the compounds.

REFERENCES

1. Walker, *J. chem. Soc.*, 1950, 193.
2. Muir and Lewis, *J. Pharm. Pharmacol.*, 1959, **11**, Suppl., *91 T.*
3. Barlow and Vane, *Brit. J. Pharmacol.*, 1956, **11**, 198.
4. Wien, *Arch. int. Pharmacodyn.*, 1954, **97**, 395.
5. Doyle and Stove, 1958, British Patent No. 800,963.
6. Wien, Mason, Edge and Langston, *Brit. J. Pharmacol.*, 1952, **7**, 534.
7. Burn and Dale, *J. Pharmacol.*, 1915, **25**, 417.
8. Paton and Zaimis, *Brit. J. Pharmacol.*, 1951, **6**, 155.

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

It was suggested that the higher charge density round the N atom due to its small atomic radius, is the reason for the physiological activity being more affected by change in substitution on this atom than on the S atom.

THE SPECTROPHOTOMETRIC DETERMINATION OF CERTAIN ALKALOIDS AND APPLICATION TO PHARMACEUTICAL PREPARATIONS

By A. H. J. Cross, D. McLaren* and S. G. E. Stevens

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A method is described for the determination of atropine, hyoscyamine, quinine, quinidine, brucine, strychnine and physostigmine by formation of the picrate of the alkaloid in an aqueous buffer at pH 7 followed by extraction of the picrate into chloroform. The picric acid is re-extracted into an alkaline buffer at pH 11 and the absorption measured at 3550 Å. The method has been applied to injections, tablets and other pharmaceutical preparations. The results are in agreement with those obtained by the official methods. The advantages of the method include its usefulness when dealing with small samples and the shorter assay time compared with classical methods.

Richter[1] devised a method for the estimation of higher amines and alkaloids based on the fact that picric acid gave little colour in 50 per cent chloroform-toluene or chloroform-light petroleum solution while amine picrates were strongly coloured, and used this method[2] to determine amphetamine, ephedrine and methylisomyn in urine. Gad[3] applied the method to the determination of amphetamine in organs, tissue and excreta after separation of the base by steam distillation from an alkaline preparation of the specimen. Page and Hopewell[4] applied the method to long-chain amines but obtained poor recoveries. Trautner, Neufeld and Rodwell[5] titrated atropine, hyoscyamine and hyoscine in chloroform solution with a standard solution of picric acid in chloroform and found that whereas atropine and hyoscyamine picrates were soluble, hyoscine picrate was only slightly soluble in chloroform. Semenicheva[6] determined atropine sulphate in eye drops by treating a neutral solution of the alkaloid with sodium picrate and extracting the alkaloid picrate into chloroform. After separation and removal of the chloroform, the residual picrate was reduced with sodium sulphide solution and the colour produced was compared with standards prepared from picric acid reduced in the same way. Cunningham, Dawson and Spring[7] determined the molecular weight of hydrocarbons and bases by preparation of the corresponding picrates and determination of the absorption in ethanolic solution at 3800 Å.

In the method now reported the picrates of certain alkaloids were formed in an aqueous buffered solution and then extracted into chloroform. After separation of the chloroform solution, the picrate was decomposed and the picric acid extracted into an alkaline aqueous buffer leaving the base in the chloroform. The absorption was measured at 3550 Å.

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METHOD

Reagents

1 *per cent w/v solution of sodium picrate in water.* Sodium picrate is prepared and freed from picric acid. "Analar" picric acid, received as a paste containing about 30 per cent of water for safety, was assayed and the calculated equivalent of sodium hydroxide was added to a hot solution of 50 g. of the paste in 500 ml. of water. The solution was evaporated to about 300 ml. and then allowed to cool. The precipitated sodium picrate was collected on a buchner funnel, was sucked dry and washed several times with chloroform. The solid was dried *in vacuo.*

pH 7 *Buffer solution.* 10 g. potassium dihydrogen phosphate was dissolved in 1 litre of water and adjusted to pH 7·0 $\pm$ 0·05 by the addition of 30 per cent w/v aqueous sodium hydroxide solution.

pH 11 *Buffer.* 1 g. of tribasic sodium phosphate was dissolved in water to 1 litre. The pH should be between 11·2 and 11·5.

To 25 ml. of chloroform (Reagent grade) in a 125 ml. separator was added exactly 20 ml. of a solution of the base in pH 7 buffer followed by 3 ml. of the 1 per cent sodium picrate solution. After vigorous shaking the layers were allowed to separate completely, making sure that no aqueous phase was trapped in the constricted portion of the separator. The chloroform layer was run into a second separator, leaving 0·2 to 0·3 ml. in the first to ensure that no aqueous phase was transferred. The extraction was repeated with two further 25 ml. portions of chloroform.

The bulked extracts were shaken with 40 ml. of pH 11 buffer solution and after complete separation, the chloroform layer was run off. The aqueous phase was transferred to a 100 ml. graduated flask, the separator rinsed with more buffer and the bulked extract and washings diluted to 100 ml. with buffer.

The absorption of the resulting solution was determined at 3550 Å in a Hilger Uvispek in a 1 cm. silica cell using a slit setting 9 (equivalent to 5 Å bandwidth) and a similar cell containing pH 11 buffer as the blank.

A standard curve relating concentration to absorption at 3550 Å was prepared for each base by carrying known amounts of base dissolved in 20 ml. of pH 7 buffer through the process described. Blank values obtained by using 20 ml. of pH 7 buffer in place of the base solution were very low (0·002 to 0·006 absorption in a 1 cm. cell).

When a greater sensitivity was required, the final extract was diluted to 50 ml. and either 2 or 4 cm. cells were used for the measurement of the absorption.

Various preliminary manipulations were used to enable alkaloids to be determined in pharmaceutical preparations. Simple solutions such as injections or guttae were diluted with pH 7 buffer so that 20 ml. contained an appropriate quantity of alkaloid. With tablets containing alkaloids of adequate solubility at pH 7, a suitable weight was shaken with buffer and diluted to a known volume. After filtration and dilution of the filtrate with buffer, a 20 ml. aliquot was used in the assay.

Tablets of quinine or quinidine salts were powdered and shaken with 40 ml. of 0·5 N hydrochloric acid for 20 minutes. After dilution to 500

ml. with water, a 10 or 20 ml. aliquot of the filtrate was diluted with pH 7 buffer, and sufficient N sodium hydroxide solution was added to just neutralise the hydrochloric acid present before making up to 100 ml. with more buffer. 20 ml. of the final solution was used for the assay.

TABLE I

APPLICATION OF THE METHOD TO ALKALOID BASES OR SALTS

Base	mg. used (range)	Number of results	Mean O.D.* per mg.	Standard deviation
Atropine (sulphate)	0·412–1·648	4	0·244	0·0014
Hyoscyamine (sulphate)	0·441–1·765	4	0·239	0·0020
Quinine (base) hydrochloride and sulphate	0·842–2·148	8	0·422	0·0056
Quinidine (sulphate)	0·823–3·291	5	0·421	0·0028
Brucine (base)	0·669–2·674	4	0·341	0·0057
Strychnine (base)	0·303–1·211	4	0·365	0·0030
Physostigmine (salicylate)	0·404–1·616	4	0·401	0·0022

* Optical density.

TABLE II

RESULTS OBTAINED WITH PHARMACEUTICAL PREPARATIONS USING THE METHOD

Preparation	Claim	Assay†	Picrate method	Samples examined
Tab. Atrop. Sulph. B.P.	0·01 gr.	0·009 gr.	0·0097–0·0099	3
Inject. Atropine Sulph. B.P.	0·6 mg./ml.	—	0·59 mg./ml.	3
Atropine Eye Ointment B.P.	1*	0·95*	1·00–1·04*	4
Gutta Atropine Sulph. B.P.C.	1*	1·00*	0·97–0·98*	4
Guttae Physostigmine E.P.C.	0·5* Physos. Salicyl.	—	0·44–0·45*	4
Liq. Strych. Hyd. B.P.	1·0*	1·01*	0·99–1·00*	4
Ammon. Tinct. Quin. B.P.C.	1·56–1·76* quinine	—	1·62*	1
Tab. Quin. Sulph. B.P.	5 gr.	4·63 gr.	4·66 gr.	1
Tab. Quin. Hydrochlor. B.P.	5 gr.	4·86 gr.	4·75–4·81 gr.	4
Tab. Quin. Bisulph. B.P.	(*a*) 5 gr. (*b*) 5 gr.	— 4·75 gr.	4·54–4·66 gr. 4·90–4·91 gr.	3 2
Tab. Quin. Bisulph. B.P.	(*a*) 2 gr. (*b*) 2 gr.	— 1·98 gr.	1·91 gr. 1·98 gr.	1 1
Tab. Quinidine Sulph. B.P.	5 gr.	5·09 gr.	4·93–5·07 gr.	4
Proprietary tonic Ref—2284	10·9 mg. strychnine per 100 ml.	—	10·9–11·2 mg.	3
Ref—2285	"	—	10·6–10·8 mg.	3
Ref—2319	"	10·5 mg.	11·2–11·3 mg.	2
Ref—2329	"	10·4 mg.	11·1–11·3 mg.	2

* All per cent.

† The assay method used was either the official one or extraction of the alkaloid was by a "classical" technique.

Sufficient 0·5 N hydrochloric acid to neutralise the ammonia was added to 5 ml. of the ammoniated tincture of quinine before dilution to 100 ml. with water. 10 ml. of the dilution was made up to 100 ml. with pH 7 buffer and 20 ml. of this solution was used for the determination.

Atropine eye ointment (0·5 g.) was dissolved in chloroform to 100 ml. and a 25 ml. aliquot was transferred to a separating funnel. 20 ml. of pH 7 buffer and 3 ml. of 1 per cent sodium picrate solution were added. After shaking and separation of the chloroform layer, the extraction of the alkaloid picrate was continued with a further 2 × 25 ml. of chloroform. The assay was completed by the general method.

A similar modification was applied to 100 ml. of a proprietary strychnine tonic which also contained sodium and calcium glycerophosphates and phosphates and colouring agents. Sufficient sodium hydroxide solution was added to make the mixture alkaline, and the alkaloid was extracted with 3 × 25 ml. of chloroform. 20 ml. of pH 7 buffer and 3 ml. of 1 per cent sodium picrate solution were added to the bulked chloroform extracts and after vigorous shaking, the separated chloroform layer was shaken with 40 ml. of pH 11 buffer. The assay was completed as before.

Results

The method was applied to several alkaloids using either the bases or salts as set out in Table I.

Table II lists the results obtained with some pharmaceutical preparations. Most preparations were also assayed by the official method or a "classical" extraction method ending with either the weighing or titration of the extracted alkaloid was used.

Discussion

Any base whose picrate is largely undissociated at pH 7 and for which the partition coefficient between the aqueous phase and chloroform favours extraction into chloroform should be amenable to determination by the technique described. It has proved especially useful for the determination of small quantities of atropine, hyoscyamine and strychnine in a variety of pharmaceutical forms, and has given more reproducible

TABLE III

Apparent molecular extinction coefficients for alkaloidal picrates

Alkaloids	Apparent ε of picrate
Atropine	14,080
Hyoscyamine	13,800
Quinine	13,700
Quinidine	13,660
Brucine	13,410
Strychnine	12,200
Physostigmine	11,050

results than similar techniques based on the sulphonphthalein dye complex method of Brodie[8] or the many published modifications thereof. Hyoscine did not give satisfactory recoveries, probably because of the very low solubility of its picrate in chloroform reported by Trautner and others[5]. The method was also applied to nicotine, emetine and ephedrine but the results did not warrant its use for the determination of these alkaloids. For the alkaloids reported, the extraction of the base-picrates into chloroform was checked by assaying separately each of the three

25 ml. extracts and in every case more than 95 per cent of the extractable picrate was removed in the first 25 ml. The picric acid was completely extracted from the chloroform solution by one extraction with 40 ml. of the alkaline buffer leaving the base in the chloroform layer.

The apparent molecular extinction coefficients for the alkaloid picrates listed in Table III may be calculated. Picric acid has a molecular extinction coefficient of 14,140 in pH 11 buffer. The dissociation of the alkaloid picrates of atropine, hyoscyamine, quinine, quinidine and brucine, was largely depressed in the presence of the large excess of picrate ion. For strychnine, only about 86 per cent and for physostigmine only 78 per cent of the alkaloid was present as the picrates.

Provided the stated conditions for the assay were adhered to this did not represent a drawback since the degree of dissociation was constant. By using a higher concentration of sodium picrate in the method the dissociation of the picrates could be further suppressed, but the blank values obtained were too large.

References

1. Richter, *Lancet*, 1938, **1**, 1275.
2. Richter, *Biochem. J.*, 1938, **32**, 1763.
3. Gad, *Arch. exp. Path. Pharmak.*, 1940, **196**, 25.
4. Page and Hopewell, *Analyst*, 1945, **70**, 17.
5. Trautner, Neufeld and Rodwell, *Aust. Chem. Inst. J. Proc.*, 1948, **15**, 55.
6. Semenicheva, *Aptechnoe Delo.*, 1953, **2**, 18.
7. Cunningham, Dawson and Spring, *J. chem. Soc.*, 1951, 2305.
8. Brodie, Udenfriend and Dill, *J. biol. Chem.*, 1947, **168**, 335.

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

A mixture of alkaloids which formed picrates would give a total assay figure, but the number of alkaloids forming picrates in chloroform is limited. In general, phenolic preservatives would not interfere with the method, as their picrates would favour the aqueous phase. The method was satisfactory for the determination of small quantities of the atropine alkaloids, but was especially designed for forensic analysis.

A NEW SYNTHESIS FOR 1-AMINOHYDANTOIN AND NITROFURANTOIN

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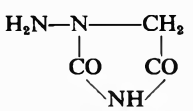
Received June 1, 1959

A new synthesis for 1-aminohydantoin and nitrofurantoin is described. Derivatives of 1-aminohydantoin were prepared by condensation of semicarbazones with ethyl monochloroacetate in presence of sodium alkoxide in dry ethanol. The nature of this reaction is discussed and semicarbazones were shown to react as nucleophilic anions.

Nitrofurantoin, 1-(5′-nitro-2′-furfurylideneamino)hydantoin, is an important antibacterial drug in the treatment of infections of the urinary tract. It is prepared by condensing 5-nitro-2-furaldehyde or its diacetate with 1-aminohydantoin. The furan derivative may be prepared in good yield from furfural by nitration[1] but, until recently, 1-aminohydantoin (I) was obtained in relatively low yield by a rather slow process from monochloroacetic acid through hydrazino-monoacetic acid and 2-semicarbazido-acetic acid. In these reactions, the yield of 1-aminohydantoin is low mainly because of the formation of a large proportion of hydrazinodiacetic acid from the condensation of hydrazine and monochloroacetic acid. Even when 6 times excess of hydrazine is used, the yield of 1-aminohydantoin is only about 35 to 40 per cent[2] when allowance is made for hydrazine recovered by distillation. Accordingly, an improved synthesis for 1-aminohydantoin was desirable.



(I)

Simple semicarbazones such as benzaldehyde or acetone semicarbazone condense with ethyl monochloroacetate to form aldehydic or ketonic derivatives of 1-aminohydantoin in good yield. These derivatives are easily hydrolysed by mineral acids to give 1-aminohydantoin which may be condensed with 5-nitro-2-furaldehyde or its diacetate to give nitrofurantoin in about 60 per cent yield based on starting hydrazine compared with 35 to 40 per cent by the best previously known route. In addition, the new process is quicker and involves no hydrazine recovery or other potentially hazardous stage.

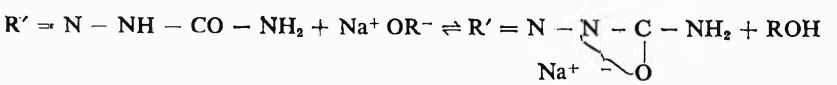
The improved yield of 1-aminohydantoin is due mainly to the selection of a starting hydrazine derivative which contains only one residual hydrogen atom for substitution. Usually, hydrazine forms preferentially gem-disubstituted derivatives with alkylating agents since mono-alkyl hydrazines are more basic than hydrazine[3,4]. But, in semicarbazones, two

of the hydrazine hydrogen atoms are replaced by an aldehydic or ketonic residue which is easily removed after the condensation to hydantoin is complete, and a third by an amide group which takes part in the reaction to form the hydantoin structure. However, semicarbazones are not basic and do not react spontaneously with chloroacetic acid derivatives and so reaction conditions have to be used to form highly reactive semicarbazone anions. The reaction is discussed further below.

The use of semicarbazones in the synthesis of *N*-aminoheterocycles is novel. Other applications of this type of reaction have been investigated and will be reported soon.

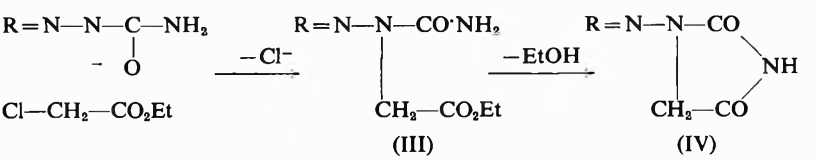
The Reaction between Semicarbazones and Ethyl Monochloroacetate

The basis of the new reaction is the formation of strongly nucleophilic semicarbazone anions (II) by treating the semicarbazone with a strong base under anhydrous conditions, for example with a sodium alkoxide in dry alcohol.



(II)

The semicarbazone ion reacts with added ethyl monochloroacetate, by a nucleophilic attack on the α-carbon of the ester with formation of a C–N bond and elimination of chloride ion, and by an ester-amide condensation resulting in the formation of a second C–N bond with elimination of ethanol and completion of the hydantoin structure (IV). The reaction is strongly exothermic and sodium chloride precipitates almost immediately the ester addition is begun. No intermediate 2-semicarbazidoacetic ester (III) could be isolated presumably because in it, the ester and amide groups are held close together in an environment ideal for their condensation.



(III) (IV)

The hydantoin formed, being a relatively strong acid, reacts with the sodium derivative of the semicarbazone to give a sodium hydantoin and free semicarbazone. Accordingly, the theoretical relative proportion for the reaction are semicarbazone (1 mole), sodium alkoxide (2 moles) and ethyl monochloroacetate (1 mole), but for best yields, not all of the ethoxide may be added at once since the excess would react readily with the chloro-ester to give an ethyl alkoxyacetate. The following scheme shows the theoretical optimum method of addition.

Semicarbazone (1 mole) + sodium alkoxide (1 mole)
↓ ethyl monochloroacetate (½ mole)
Sodium hydantoin (½ mole) + semicarbazone (½ mole) + sodium chloride (½ mole)
↓ 1. Sodium alkoxide (½ mole) 2. Ethyl monochloroacetate (¼ mole)
Sodium hydantoin (¼ mole) + semicarbazone (¼ mole) + sodium chloride (¼ mole)
↓ 1. Sodium alkoxide (¼ mole) 2. Ethyl monochloroacetate (⅛ mole)
Sodium hydantoin (⅛ mole) + semicarbazone (⅛ mole) + sodium chloride (⅛ mole)
↓ 1. Sodium alkoxide (⅛ mole) etc.

In practice, the scheme was followed to the second ester addition and thereafter the alkoxide and ester were added in alternate estimated equivalent additions since the quantities involved became small. Also, whilst the scheme allows theoretical conversion of semicarbazone to a 1-aminohydantoin derivative, lower yields are obtained because of the competing side-reaction between the alkoxide and chloroester yielding an ethyl alkoxyacetate. The yield of hydantoin obtained depends on the relative concentrations of semicarbazone and alkoxide ions and their relative reactivities towards the chloroester.

With benzaldehyde semicarbazone the reaction product was the sodium derivative of 1-benzylideneaminohydantoin. 1-Benzylideneaminohydantoin was easily isolated by acidification of the reaction mixture and separated from unchanged benzaldehyde semicarbazone by treatment with aqueous alkali in which the semicarbazone was sparingly soluble. 1-Aminohydantoin hydrochloride was obtained by hydrolysis of the benzylidene derivative with hydrochloric acid and was converted to free 1-aminohydantoin by treatment with an equivalent of aqueous alkali or with sodium methoxide in dry alcohol. With acetone semicarbazone the reaction product was the sodium salt of 1-isopropylideneaminohydantoin. The acid form of this hydantoin was not readily isolated but was converted to 1-benzylideneaminohydantoin or nitrofurantoin by reaction with benzaldehyde or 5-nitro-2-furaldehyde respectively in acid solution.

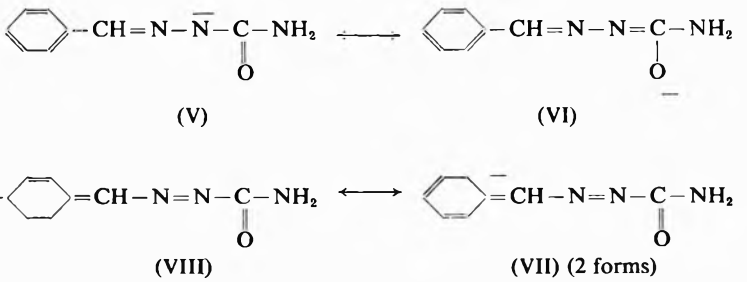
Semicarbazone Anions

The following evidence confirms the existence of semicarbazone anions.

Acetone and benzaldehyde semicarbazones are much more soluble in a solution of sodium ethoxide in dry ethanol than in ethanol alone.

Benzaldehyde semicarbazone could be titrated partially as an acid with sodium methoxide in ethylenediamine using azo-violet as indicator[5] but, under these conditions, acetone semicarbazone showed little or no acidity and is, therefore, a weaker acid than the benzaldehyde derivative. This would be expected because of the inductive effect (+I) of the isopropylidene group which partially satisfies the electron demand of the 2-nitrogen of acetone semicarbazone and so its substituent hydrogen atom ionises less readily, and because of the greater possibility for resonance and,

therefore, stability in benzaldehyde semicarbazone anion. Resonance forms are shown in V to VIII.



(V) (VI)

(VIII) (VII) (2 forms)

Acetone semicarbazone, being a weaker acid than benzaldehyde semicarbazone, should yield a lower concentration of anion than benzaldehyde semicarbazone under similar conditions, and, therefore, a lower yield of hydantoin in its reaction with ethyl monochloroacetate. The yields obtained, isolated as 1-benzylideneaminohydantoin in each case, were 60 to 65 per cent from acetone semicarbazone and 70 to 74 per cent from benzaldehyde semicarbazone. However, this difference might also be attributed to different reaction rates due to steric effects in the two ions.

The absorption spectrum of benzaldehyde semicarbazone showed a bathochromic shift when the solvent was changed from super-dry ethanol (λ max 283 mμ, ϵ 22,900) to 1·2N sodium methoxide in the same ethanol (λ max 323 mμ, ϵ 15,000) thus indicating probable anion formation.

The Effect of Water on the Reaction

Absence of water is essential for good yields in the new synthesis, the yield of hydantoin falling rapidly as the water content is increased. For good results the solvent should not contain more than about 0·1 per cent of water because semicarbazones, being very weak acids, do not form anions in the presence of water. In proof of this, it was shown that benzaldehyde semicarbazone did not exhibit a bathochromic shift in 0·1N sodium methoxide in methanol containing 0·2 per cent of water. Also, it is well known that in ester-amide condensation reactions good yields depend on the absence of water, the preparation of barbituric acids from malonic esters and urea being a good example[6].

Experimental

Melting points are uncorrected.

Ultra-violet spectra were determined with a Beckman DK2 recording spectrophotometer.

1-*Benzylideneaminohydantoin*

From 1-*Aminohydantoin hydrochloride.* Benzaldehyde (10·6 g.) was added to a solution of 1-aminohydantoin hydrochloride (15·2 g.) in a mixture of water (150 ml.) and ethanol (150 ml.). A white crystalline

solid separated almost immediately, was filtered off, washed with water and dried at 80° *in vacuo* to give 1-benzylideneaminohydantoin (19·4 g.; 95·5 per cent), m.p. 252 to 254°. Needles from 50 per cent v/v aqueous ethanol, m.p. 254 to 255°. (Traube and Hoffa[7] give 244°, Carrara and others[8] 249 to 250° and Uota and others[9] 245°.)

From benzaldehyde semicarbazone. Benzaldehyde semicarbazone (16·3 g.) was dissolved with heating and stirring in a solution of sodium (2·3 g.) in dry ethanol (50 ml.). Ethyl monochloroacetate (6·2 g.) was added at such a rate as to maintain refluxing without external heating (3 to 4 min.), then the mixture was refluxed for 10 minutes. A solution of sodium (1·15 g.) in dry ethanol (25 ml.) was added and the mixture heated at reflux and stirred for about one minute. Ethyl monochloroacetate (3·1 g.) was added as before and the mixture refluxed for 10 minutes. Sodium (1·15 g.) dissolved in dry ethanol (25 ml.) and ethyl monochloroacetate (3·1 g.) were added in that order in alternate small estimated equivalent additions refluxing briefly after each addition. Finally, the mixture was refluxed for 30 minutes and then most of the ethanol removed by distillation to give an alkaline pasty solid. Concentrated hydrochloric acid (15 ml.) mixed with water (80 ml.) and crushed ice (100 g.) was added, the mixture stirred and the insoluble white solid filtered off, washed and dried at 100 to 110° to give 18·5 g. solid, m.p. 228 to 240°. The solid was triturated with aqueous sodium hydroxide solution (250 ml. of 5 per cent w/v) and then filtered. The filter residue was slightly impure benzaldehyde semicarbazone (2·9 g.), m.p. 218 to 222°. The filtrate was acidified with hydrochloric acid (100 ml. of 15 per cent w/v HCl) and the precipitated white solid filtered off, washed with water and dried at 100 to 110° to give crude 1-benzylideneaminohydantoin (15·2 g.; 75 per cent) m.p. 246 to 250° which crystallised from aqueous acetic acid (250 ml. of 50 per cent v/v) giving 1-benzylideneaminohydantoin as colourless rods (14·2 g.; 70 per cent), m.p. 254 to 255°. Found: equiv. wt. 209 (sodium methoxide in benzene-methanol solution), 204 (sodium methoxide with dimethyl formamide as solvent). Required: equiv. wt. 203. The melting point was not depressed in admixture with 1-benzylideneaminohydantoin prepared from 1-aminohydantoin hydrochloride and the infra-red absorption spectra were identical for the two samples.

From Acetone Semicarbazone

This preparation was practically identical with that from benzaldehyde semicarbazone to the removal of ethanol stage substituting acetone semicarbazone (11·5 g.) for the benzaldehyde semicarbazone. No separation of 1-isopropylideneaminohydantoin occurred when the reaction mixture was acidified. Benzaldehyde (11·0 ml.) was added with ethanol (50 ml.) and the mixture heated to about 60° then cooled to 30° and filtered. The white filter residue was washed with water and dried to give slightly impure 1-benzylideneaminohydantoin (12·4 g.; 61 per cent) m.p. 248 to 252°. This material was almost completely soluble in aqueous sodium hydroxide or carbonate solution. After crystallisation

from aqueous ethanol the melting point was 254 to 255° which was not depressed when the sample was mixed with a known sample of 1-benzylideneaminohydantoin.

Nitrofurantoin: 1-(5′-*Nitro*-2′-*furfurylideneamino*)*hydantoin*

From 1-*Aminohydantoin hydrochloride*. A mixture of 5-nitro-2-furaldehyde diacetate (2·43 g.), 1-aminohydantoin hydrochloride (1·52 g.), ethanol (10 ml.), concentrated hydrochloric acid (5 ml.) and water (20 ml.) was refluxed for 30 minutes and then cooled. The yellow crystalline needles formed were filtered off, washed with water and dried at 80° *in vacuo* to give 1-(5′-nitro-2′-furfurylideneamino)hydantoin (2·2 g.; 91 per cent), m.p. 266 to 268° (decomp.).

From 1-*benzylideneaminohydantoin*. A mixture of 1-benzylideneaminohydantoin (2·03 g.) and dilute sulphuric acid (55 ml. of 10 per cent v/v H_2SO_4) was distilled until the distillate was free from benzaldehyde. Ethanol (10 ml.) and 5-nitro-2-furaldehyde diacetate (2·43 g.) were added and the mixture refluxed for 10 minutes and then cooled. The filtered and washed residue was dried at 80° *in vacuo* to give 1-(5′-nitro-2′-furfurylideneamino)hydantoin (2·18 g.; 92 per cent), m.p. 266 to 267° (decomp.).

From acetone semicarbazone. The preparation of 1-benzylideneaminohydantoin from acetone semicarbazone is described above. By a similar process acetone semicarbazone may be converted directly into nitrofurantoin in one reaction vessel by adding 5-nitro-2-furaldehyde diacetate instead of benzaldehyde in the final stage and refluxing for 15 minutes followed by cooling. The nitrofurantoin obtained is impure but may be purified by crystallisation from, for example, aqueous acetic acid. In one such experiment using acetone semicarbazone (11·5 g.) and later adding 5-nitro-2-furaldehyde diacetate (24·3 g.) there was obtained a crude product (16·9 g.; 67 per cent), m.p. 258 to 262° (decomp.) which after crystallisation from aqueous acetic acid (50 per cent v/v) gave 1-(5′-nitro-2′-furfurylideneamino)hydantoin (14·2 g.; 59 per cent), m.p. 266 to 268° (decomp.).

All of the above samples of nitrofurantoin showed no depression of melting point with an authentic sample and had practically identical ultra-violet and infra-red absorption characteristics.

1-*Aminohydantoin Hydrochloride*

From 2-*Semicarbazido-acetic acid*. 2-Semicarbazido-acetic acid (6·65 g.) was heated for 30 minutes at 100° with a mixture of concentrated hydrochloric acid (25 ml.) and water (10 ml.). The mixture was evaporated to near dryness with water-pump vacuum and absolute ethanol (20 ml.) was added. The white crystalline solid was filtered off, washed well with absolute ethanol and then dried to yield 1-aminohydantoin hydrochloride (6·5 g.; 86 per cent) m.p. 198 to 200° (decomp.) (Traube and Hoffa give 203° (decomp.)). Found: equiv. wt. 1. Aqueous NaOH to methyl red; 151. 2. Sodium methoxide in benzene/methanol; 75·3. Required: 1. 151·5. 2. 75·8.

From 1-*Benzylideneaminohydantoin.* A mixture of 1-benzylideneaminohydantoin (20·3 g.), concentrated hydrochloric acid (250 ml.) and water (250 ml.) was distilled until the distillate was free from benzaldehyde and then evaporated to near dryness under reduced pressure. Absolute ethanol (50 ml.) was added and the crystalline white solid filtered off, washed with ethanol and dried to give 1-aminohydantoin hydrochloride (13·3 g.; 88 per cent), m.p. 199 to 201° (decomp.).

1-*Aminohydantoin.* Sodium bicarbonate (5·55 g.) was added to a solution of 1-aminohydantoin hydrochloride (10·0 g.) in water (28 ml.). The resultant solution was cooled to 4° when colourless prisms separated, were filtered off, washed with a little water and then dried at 60° *in vacuo* to give 1-aminohydantoin (5·0 g.; 65·6 per cent), m.p. 195°. (Uota and others give 193° and Carrara and others 195 to 196°.) Found: equiv. wt., sodium methoxide in benzene/methanol 117. Required: equiv. wt. 115.

A mixture of 1-aminohydantoin hydrochloride (15·1 g.), sodium methoxide (5·4 g.) and absolute ethanol (200 ml.) was refluxed for 15 minutes and then filtered to remove sodium chloride. From the filtrate crystallised a colourless solid as rhombic prisms was filtered off, washed with ethanol and dried at 60° *in vacuo* to give 1-aminohydantoin (8·85 g.; 77 per cent), m.p. 194 to 195°.

The author thanks Dr. A. H. Beckett for help and advice and Mr. A. H. J. Cross and Mr. T. H. Watts for carrying out the infra-red absorption measurements.

References

1. Brit. Patent 765,898 to Norwich Pharmacal Company, 1954.
2. Brit. Patent 757,822 to Norwich Pharmacal Company, 1954.
3. Reed, *Hydrazine and its Derivatives*, R.I.C. Report No. 5, 1957.
4. Audrieth and Ogg, *The Chemistry of Hydrazine*, Wiley, N.Y., 1951.
5. Beckett and Tinley, *Titration in Non-aqueous Solvents*, B.D.H. Ltd., 2nd Edn.
6. Organic Syntheses, Coll. Vol. II, 60.
7. Traube and Hoffa, *Ber.*, 1898, **31**, 162.
8. Carrara, Ettore, Fava and Vecchi, *Gazz. chim. ital.*, 1953, **83**, 609.
9. Uota, Takai, Tanizaki and Yokoi, *J. Pharm. Soc., Japan*, 1954, **74**, 697.

After the Author presented the paper there was a DISCUSSION.

A LIGHT-SCATTERING STUDY OF LYSOLECITHIN SOLS

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Received May 22, 1959

Sols of four samples of lysolecithin have been studied by means of a light-scattering apparatus. The results of a large number of measurements have been analysed statistically. They indicate that the mean molecular weight of the micelles in the sols is 92,400, the experimental error in this estimate being 7 per cent.

Light-scattering measurements can be used to provide information about the size and shape of macromolecules, a method we have applied to the further studies of the physical chemistry of phosphatide sols.

The theoretical foundations of light-scattering phenomena were laid by Rayleigh in 1871[1]. Since Debye's[2,3] recent development of the theory the method has made a large contribution to the understanding of biological substances, high polymers and other macromolecules.

The apparatus constructed in our laboratory is based on that described by Hughes, Johnson and Ottewill[4]; it was calibrated with Ludox (a silica sol) and several organic solvents. The apparatus which is shown in plan, in Figure 1, was then tested by examining aqueous sols of some fractionated proteins and non-aqueous sols of high polymers; all gave molecular weights in agreement with values obtained by other workers using this technique. The instrument was found to be suitable for molecular weight determinations in the range 5,000 to 500,000.

Before commencing a series of light-scattering experiments on phosphatide sols we have examined the reproducibility of determinations of the molecular weight of lysolecithin by making a large number of light-scattering measurements with aqueous sols of four different preparations of this substance.

Experimental

Four different samples of lysolecithin (A, B, C and D) were prepared by the action of viper venom on hen egg lecithin. Aqueous sols of this substance were obtained as previously described[5] and centrifuged at 8,300 *g*. Before the light-scattering measurements were made each sol was filtered through a fine sintered glass frit (1 μ) under a pressure of 10 cm. of mercury, to remove traces of dust.

The sol was transferred to a rectangular glass cell ($5 \times 5 \times 1$ cm.^{3}) and placed in a thermostat jacket in the path of a beam of parallel light of wavelength 4,358 Å. The light was scattered in all directions by the micelles in the sol and the intensity of scattering at 90° to the incident beam was measured by means of an 11-stage photomultiplier tube connected directly to a mirror galvanometer. The symmetry of the light scattered about the 90° angle was also examined, all the scattered intensities being related to those obtained with a standard lead glass block.

The depolarisation factor for the scattered light at 90° was 0·0105. The dissymmetry I_{50}/I_{130} (where I is the intensity of scattered light and the subscript is the angle to the incident beam) for the samples A, B and D was 1·08 whilst for the sample C it was 1·17. All measurements were taken at a temperature of 20°.

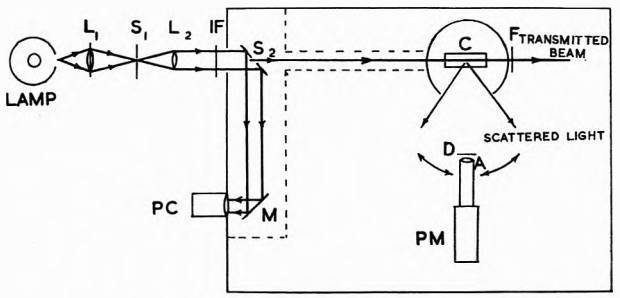

FIG. 1. Diagrammatic sketch of apparatus.

L_1 L_2 = Lenses. S_1 S_2 = Slits.
IF = Interference filter isolating $\lambda = 4358$Å.
F = Neutral density filter.
D = Polaroid disc for measuring depolarisation of scattered light.
A = Aperture. M = Mirror .
C = Cell immersed in thermostat-jacket.
PC = Photocell (connected via switch to galvanometer) for measuring intensity of incident beam.
PM = 11-stage photomultiplier (connected via switch to galvanometer) for measuring intensity of scattered light.

The specific refractive index increment of lysolecithin in water was determined by means of a Rayleigh interference refractometer modified for monochromatic light as described by Bauer[6]. This quantity, included in the constant K of the equation below, was found to be 0·1377.

METHOD

For approximately spherical molecules, the relation between light scattered at 90° to the incident beam, characterised by the term R_{90}, and concentration X per cent w/v is given by the equation[7]: $\frac{K.X}{R_{90}} = \frac{1}{M_w} + BX$, where K is a constant containing optical terms for a given system. R_{90} is calculated from the light scattered at 90° corrected for the scattering attributed to pure solvent and for depolarisation effects. Corrections for the dissymmetry and concentration of unassociated molecules[8] were small and are not included. M_w is the weight-average molecular weight of the solute and B is a correction term for non-ideality,—in ideal solutions it is zero. If $K.X/R_{90}$ is denoted by Y, then the plot of Y/X should give a straight line which, when extrapolated to X = O, gives an intercept Y_o equal to the reciprocal of the molecular weight of the solute.

RESULTS

When experimental values of Y were plotted against X they showed a random variation about the best straight line through the points (see Fig. 2). This is attributed to experimental error in the scattering measurements; relative to this, the error in the values X of concentration may

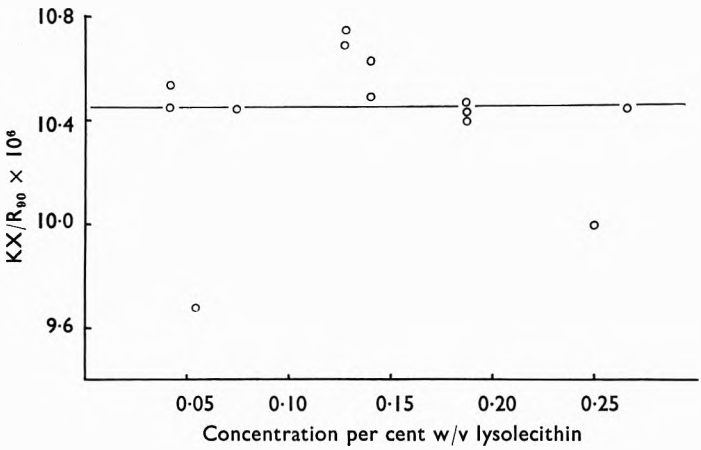

FIG. 2. Light-scattering of lysolecithin (B) in water at 20°.

be considered to be negligible. To obtain the best value of Y_o a regression analysis was made; this had the advantage of giving limits of error for the final estimate of molecular weight.

Y/X correlation coefficients were first computed for all the results obtained with each of the four samples of lysolecithin and these are given in Table I. For samples A, B and D the correlations were not significant,

TABLE I

Y/X CORRELATION AND REGRESSION COEFFICIENTS

Sample	N	Range of X	r	Theoretical r (P = 0·95)	Significance of r	b
A	53	0·06–1·00	−0·063	0·27	none	−0·306
B	13	0·04–0·25	−0·079	0·56	none	−0·403
C	13	0·02–0·12	0·793	0·56	significant	20·65
D	37	0·002–0·27	0·197	0·33	none	2·646

N is the number of scattering measurements made with each sample. X is the concentration of lysolecithin in g./100 ml. Y is the scattering quantity $K.X/R_{90}$ (see text). r is the correlation coefficient calculated from the results. The theoretical values of r for a probability of 0·95 were obtained from Fisher and Yates tables[9]. b is the regression coefficient[10] Y/X.

meaning that the slopes of the regression lines were not significantly different from zero. In these samples, therefore, the simple mean $\bar{Y}$ of the values of Y could be taken to be the reciprocal of the molecular weight. However, this treatment probably underestimates the experimental error in M_w and so in these samples the regression coefficient was also calculated and the extrapolated value $Y_o = \bar{Y} - b\bar{X}$ found. The values of Y_o were little different from $\bar{Y}$, as expected by the insignificant correlation of Y and X, but their limits of error which take into

account both the variance of Y and of the regression coefficient (see Appendix), are somewhat wider and probably more realistic.

The molecular weight estimates for samples A, B and D agree within their limits (see Table II) and so a grand mean total could be calculated. The limits for this were found to be 86,700 and 98,600 at P = 0·99 with a grand mean of 92,400. The experimental error in these molecular weight estimates from light-scattering appears to be about 7 per cent.

For sample C, different results were obtained. The Y/X correlation was clearly significant (Table I) and so $\bar{Y}$ could not be used in place of Y_o for estimating the molecular weight. The distribution of the X, Y points about the regression line was wider than in the previous samples

TABLE II

ESTIMATES OF MOLECULAR WEIGHT OF LYSOLECITHIN MICELLES

Sample	Section (i)			Section (ii)		
	$\bar{Y} \pm$ L.E. $\times 10^4$	M_w from $\bar{Y}$	Limits	$Y_o \pm$ L.E. $\times 10^4$	M_w from Y_o	Limits
A	11·37 ± 0·49	88,000	84,300–91,900	11·48 ± 0·80	87,100	81,400–93,600
B	10·41 ± 0·29	96,000	93,500–98,800	10·46 ± 0·67	95,600	89,900–102,100
C	*r* is significant			6·34 ± 1·19	157,700	132,800–194,200
D	10·28 ± 0·39	97,300	93,700–101,000	10·06 ± 0·63	99,400	93,500–106,000

In Section (i):

$\bar{Y}$ is the arithmetic mean of the values of Y for a sample. L.E. given by $\pm t\sqrt{(V/N)}$, are the P = 0·99 limits of error for Y, where V is the variance of the values of Y about the mean and *t* is a theoretical quantity derived from tables. M_w is the reciprocal of $\bar{Y}$ and the limits are the reciprocals of ($\bar{Y}$ − L.E.) and ($\bar{Y}$ + L.E.) respectively.

In Section (ii):

Y_o is the extrapolated value of Y at X = O (see Appendix). L.E. are the limits of error for Y_o for P = 0·99, calculated as $\pm t\sqrt{(V_{Y_o})}$, the variance of Y_o is calculated as shown in the Appendix. M_w and the limits are the reciprocals of Y_o and its limiting values respectively.

and the limits for the molecular weight estimate were more than twice as wide as those for samples A, B and D. The mean molecular weight was very much higher and differed significantly from the other three, there being no overlapping of the limits. Sample C was recrystallised from a batch prepared some months earlier and it may have contained traces of fatty acids. This conclusion is supported by the observation that the dissymmetry factor for C was higher than for A, B and D. In view of the wide limits of error for the estimate of molecular weight obtained from sample C (−16 and +23 per cent) we have felt justified in omitting it from the grand mean molecular weight of 92,400.

The results for sample C illustrate the uncertainty of physical measurements on materials derived from biological sources; in such work it is desirable that many results should be obtained with different samples of the substance. The results can then be summarised by making a statistical analysis.

Acknowledgements. The authors thank Dr. R. H. Ottewill, University of Cambridge, for permitting one of us (N.R.) to use his apparatus before our own was built, and Mr. P. Barden for constructing the apparatus.

Appendix

Variance of Extrapolated Value Y_o

From Figure 3 it is seen that if b is the slope of the regression line and $\bar{X}$, $\bar{Y}$ are the means of all the values of X and Y respectively, then since the regression line always passes through the point $\bar{X}$, $\bar{Y}$

$$Y_o = \bar{Y} - b\bar{X}$$

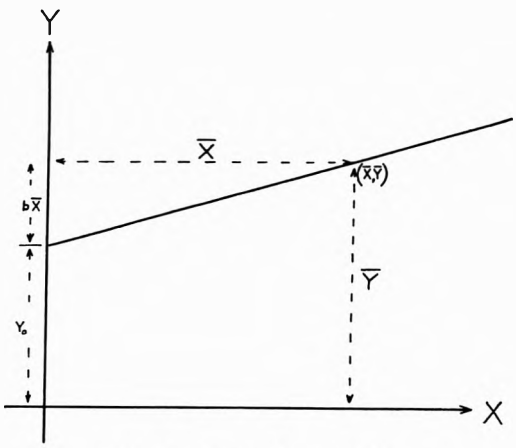

Fig. 3. Regression line Y/X.

$\bar{Y}$ is subject to random error and so is b, but the variance in X (the concentration of a solution) is small compared with the variance of values of Y; $\bar{X}$ can therefore be regarded as having no variance. The variance of Y_o is then given by $V_{Y_o} = V_{\bar{Y}} + \bar{X}^2 V_b$, both $V_{\bar{Y}}$ and V_b can be estimated from the set of values of Y and X.

References

1. Rayleigh, *Phil. Mag.*, 1871, **41**, 447.
2. Debye, *J. Appl. Phys.*, 1944, **15**, 338.
3. Debye, *J. Phys. Chem.*, 1947, **51**, 18.
4. Hughes, Johnson and Ottewill, *J. Colloid Sci.*, 1956, **11**, 340.
5. Robinson and Saunders, *J. Pharm. Pharmacol.*, 1958, **6**, 384.
6. Bauer, *Techniques of Organic Chemistry, Physical Methods*, Vol. 1, Part 11, XX, Weissburger, Interscience, N.Y., 1949.
7. Stacey, *Light-Scattering in Physical Chemistry*, Butterworths, London, 1956.
8. Robinson and Saunders, *J. Pharm. Pharmacol.*, 1958, **12**, 755.
9. Fisher and Yates, *Statistical Tables for Biological, Agricultural and Medical Research*, Oliver and Boyd, Edinburgh, 1945.
10. Saunders and Fleming, *Mathematics and Statistics*, Pharmaceutical Press, London, 1957.

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

INTERFACIAL FILMS BETWEEN BENZENE AND SOLUTIONS OF SALTS OF ARABIC ACID

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The interfacial tension between benzene and acacia, arabic acid and its salts falls rapidly for about eight hours and then more slowly. The effect on the interfacial tension is similar with the soluble salts of both monovalent and divalent metals. A tenacious film is formed in a few seconds and continues to increase in thickness with time. The evidence suggests that the film eventually formed is a substantial multilayer with many of the properties of an elastic solid. A film of this kind would account for the stability of acacia emulsions.

IN 1955 Shotton[1] investigated three methods of determining the interfacial tension of benzene : arabic acid and benzene : potassium arabate systems, choosing the sessile drop method as the most suitable as it involved less change in shape and surface area of the drop during a prolonged experiment. The values obtained were lower than by the other methods, indicating the system was more likely to be in equilibrium. This method was used to study similar systems.

EXPERIMENTAL

Apparatus

The apparatus was as described by Shotton[1].

Materials

Analar benzene was distilled to remove a trace of water and purified by crystallisation. Acacia preparations were made from a selected sample purified by precipitation from solution with absolute ethanol and dried under vacuum. Arabic acid was prepared by passing an approximately 10 per cent w/v solution down a column of a sulphonic acid resin in the hydrogen form[1]. From the arabic acid solution calcium and magnesium salts were prepared by the addition of the appropriate oxide until the solution was pH 7. The material was recovered by freeze drying until the moisture content was less than 15 per cent. Distilled water containing a little potassium permanganate was redistilled from an all-glass apparatus and collected in Pyrex bottles. This water and the benzene described had an interfacial tension of about 34·5 dynes/cm. This is close to the accepted value[2].

Method

Appropriate volumes of benzene and solution of arabic acid or its salt were prepared, a little of the other phase added and stored at 25° overnight to saturate.

An optical cell containing a slightly concave block of glass, siliconed to make it non-wettable by aqueous solutions so that a stable drop was

formed, was two-thirds filled with purified benzene. A drop of solution was then formed beneath the benzene by expelling a measured volume from an all-glass hypodermic syringe. A lid, from which depended the glass cylinder used as a size reference, was then fitted so that drop and cylinder were co-axial. Photographs were taken as soon as possible

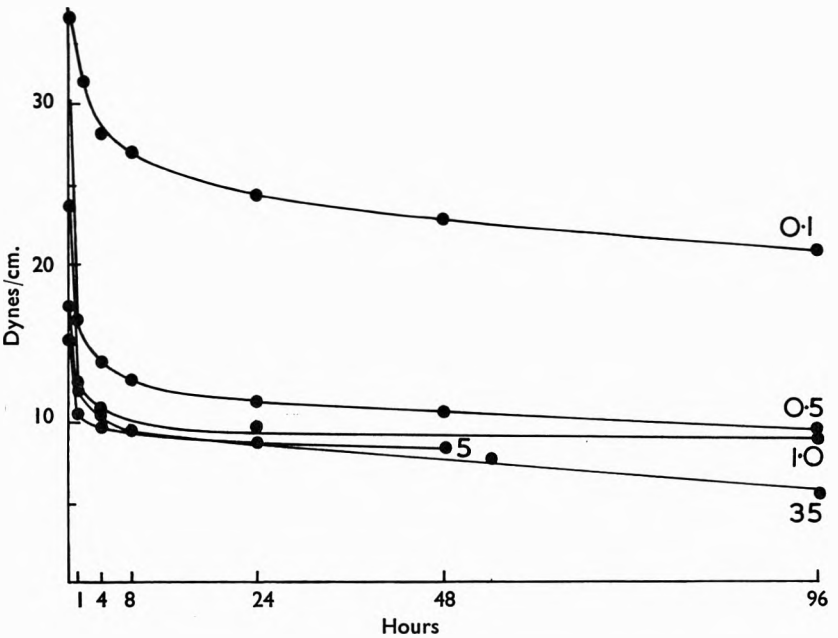

FIG. 1. Interfacial tension between benzene and solutions of natural acacia. Figures are per cent concentrations.

and at increasing intervals up to 96 hours. The image of the glass cylinder was measured and the magnification of the photograph calculated, thus making it possible to determine the actual equatorial radius of the drop and the height of the vertex above this plane. Porter's method was used to calculate the interfacial tension[3].

RESULTS

The results of the determination of the interfacial tension between benzene and solutions of crude acacia, purified acacia, arabic acid, calcium arabate and magnesium arabate are summarised in Figures 1 to 4. All the materials examined showed similar behaviour. With solutions of 1 per cent w/v and over, the interfacial tension falls rapidly for a period of about 8 hours and then the rate of decrease becomes very much slower. At 96 hours equilibrium has not been attained. Over a wide range of concentration the final interfacial tension lies in a quite small range. Solutions below 1 per cent w/v show a much slower rate of fall and the final values are substantially greater. Very dilute solutions (0·01 per cent w/v) show very little effect on interfacial tension.

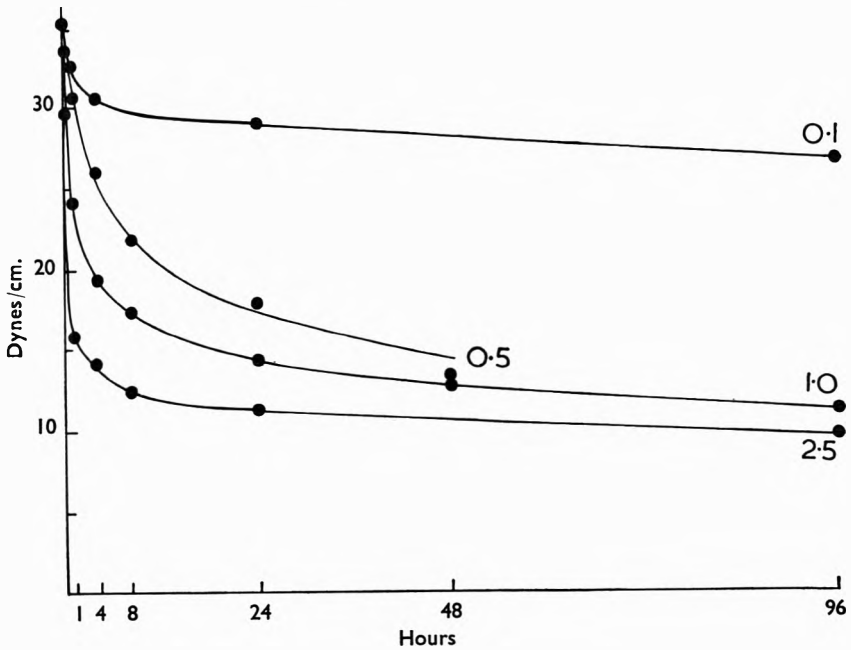

FIG. 2. Interfacial tension between benzene and solutions of purified acacia. Figures are per cent concentrations.

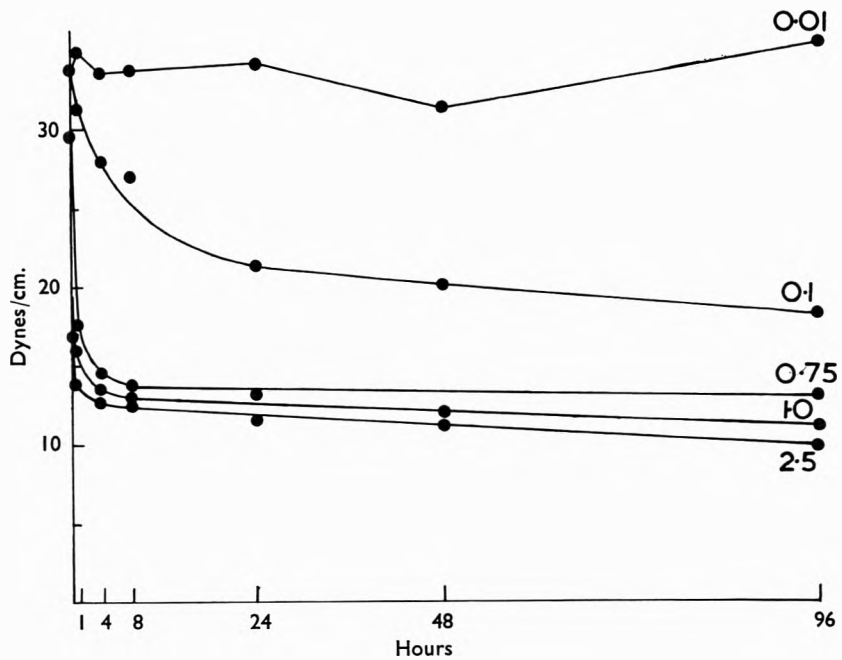

FIG. 3. Interfacial tension between benzene and solutions of calcium arabate. Figures are per cent concentrations.

Discussion of Results of Interfacial Tension Determinations

The similarity of the interfacial tension : time curves for crude acacia, purified acacia, arabic acid and its salts shows that the orientation of the molecules at an interface is little affected by the pH of the solution or the valency of the cation. The most important feature is that there is evidence of change taking place at the interface for many hours without equilibrium being reached and it is difficult to imagine that only a molecular monolayer is being built up. This is not in accord with the findings

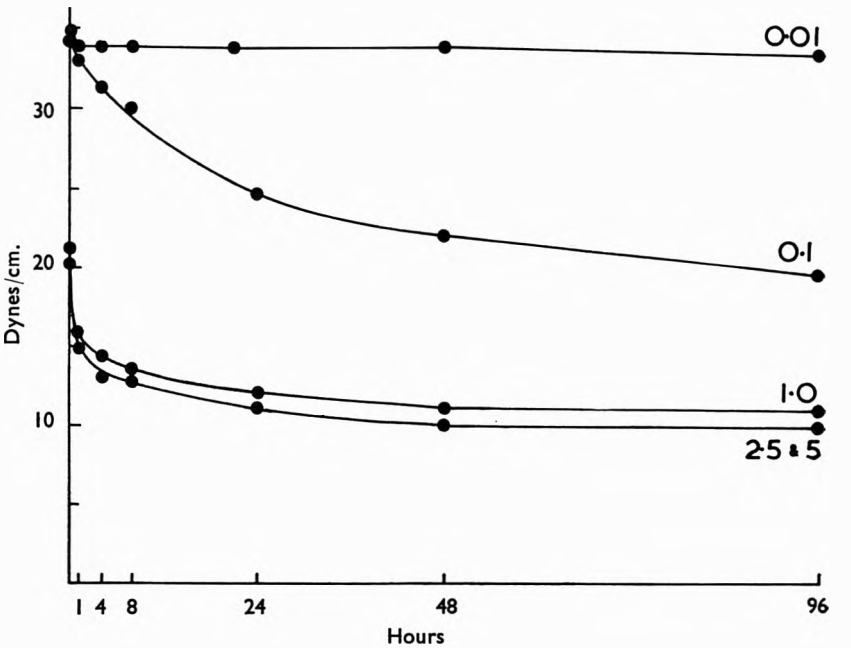

FIG. 4. Interfacial tension between benzene and solutions of magnesium arabate. Figures are per cent concentrations. The curve for 1 per cent arabic acid coincides with that for 1 per cent magnesium arabate.

of other workers[5–7], who report equilibrium to be attained in an hour or less. It is doubtful if the complex film theory for oil in water emulsions of Schulman and Cockbain[4] is applicable to this case as only one molecular species is present. However, since magnesium, calcium and potassium arabates are soluble and ionise in solution they will form a charged film. The film attains the necessary degree of condensation without complex formation.

Evidence and Discussion on the nature of the Interfacial Film

In 1909 Marshall[8] reported the existence of solid films around emulsion globules but specifically stated that acacia did not produce such films. Bancroft in 1913[9] postulated the existence of a third phase separating the oil and water phases of an emulsion. This third phase was assumed

to consist of the emulsifying agent in a hydrated form. Sellarach and Jones[10] in 1931 reported the existence of interfacial films, which were visible to the naked eye, between acacia solutions and various oils. Two years later Sellerach and others[11] described experiments in which a wire ring was forced through these films and the force required was measured. Particular attention was drawn to the great extension of the film that took place before the film broke. We consider this implies the film may be an elastic solid.

Shotton in 1955[1] reported the appearance of wrinkles on the surface of a sessile drop 4 days old when the volume of the drop was reduced and described their slow disappearance on standing, likening it to viscous flow, but such an effect could also be ascribed to the contraction of an elastic multilayer.

Behaviour of Air Bubbles at the Interface

An attempt was made to examine the film by introducing air bubbles below the interface, in the aqueous phase. A bubble introduced into a sessile drop rose to the vertex of the drop and distorted the surface according to its size, remaining there indefinitely. Bubbles of approximately 2 mm. diameter were retained below the interface for such long periods there must be a barrier present. Such a barrier must have some of the properties of a solid since a Newtonian liquid would allow bubbles to pass by viscous flow. As this barrier was distorted by the bubble at the surface it is probable the film has elastic properties, and as it retains the bubble indefinitely it does not undergo relaxation. Elasticity also implies the existence of a tensile strength, a property usually associated with solids. This solid barrier around the drop was not visible to the naked eye by either transmitted or reflected light.

An air bubble about 2 mm. diameter introduced into a drop of 35 per cent crude acacia solution which was about 5 days old produced gross distortion of the interface. The bubble remained securely in place and did not alter materially in shape for 5 days. Subsequently the bubble burst and the upper part fell to one side remaining comparatively undamaged (Fig. 5). The fragment of the bubble must be solid in order to retain its shape. For such a structure to arise it is suggested that multilayers developed on both sides of the liquid layer enclosing the air and that the surplus solution gradually drained from between these two layers until only the solid multilayers remained, so forming a rigid structure.

Air bubbles about 2 mm. diameter passed freely through a benzene: water interface. With a 5 per cent solution of potassium arabate similar bubbles passed the interface freely for about 20 seconds after its formation. After this time the bubbles rose freely to the interface where their ascent was abruptly checked and they then moved along the curved meniscus to the wall where some remained. To account for such behaviour it is necessary to postulate the existence of a solid film at the benzene: solution interface. Solutions as dilute as 0·15 per cent potassium arabate formed these films at approximately the same speed.

Benzene globules of about 4 mm. diameter when injected into the aqueous phase of a benzene:arabate system did not readily coalesce but remained indefinitely as an extremely coarse "emulsion". This suggests that each globule rapidly acquired a film of potassium arabate.

Behaviour of Drops of Potassium Arabate Solution in Benzene

Drops of arabate solutions falling through benzene also showed evidence of a solid interfacial film. A solution of 5 per cent potassium arabate was covered by a layer of benzene and with a wire loop samples of the solution were brought near to the surface of the benzene. The lens of solution was transparent and showed no structure but on gentle movement of the loop could be made to extend almost to a hollow hemisphere without detachment or permanent distortion which shows marked elasticity. The solution when detached from the loop immediately contracted, became opaque with a greyish surface and assumed a very irregular shape. As the drop fell the shape changed but did not approximate to a sphere during its descent. The impression is that an elastic membrane held stretched over the lens of the solution contracted immediately the restraint of the frame was removed until the interfacial layer became solid but not rigid. Inside, the liquid solution attempted to assume a spherical shape but was prevented from doing so because it was enclosed in a solid sac whose surface area was much greater than that of a spherical

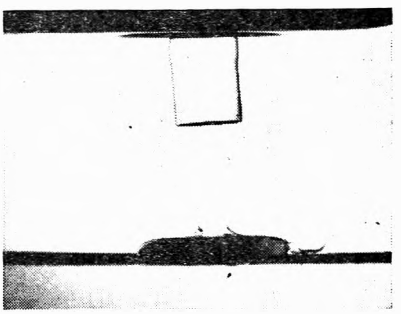

FIG. 5. Debris of rigid film formed around an air bubble at the benzene/acacia interface.

FIG. 6. Drop of potassium arabate solution, 5 per cent, falling through benzene.

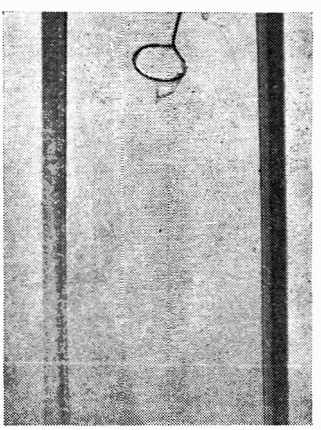

FIG. 7. Solid film of potassium arabate.

drop derived from the lens of solution held on the loop (Fig. 6). Occasionally after the detachment of the lens of solution a portion of the interfacial film was left adhering to the wire. Such a structure must be a solid (Fig. 7).

When the benzene was replaced by light liquid paraffin which is much more viscous, it was possible to manipulate the drop into any desired shape which then remained almost unchanged whilst falling through the oil. Using light liquid paraffin and water it was possible to produce spheroidal drops only.

Evidence of the existence of a tenacious or solid interfacial film may be found on interfaces only minutes old at a stage when sessile drop measurements show that the interfacial tension is still quite high. The existence of the film does not therefore depend on a very low interfacial tension being attained.

Pharmaceutical emulsions may be made with acacia in a very few minutes when benzene: 35 per cent acacia systems have an interfacial tension of about 20 dynes/cm. so emulsion formation cannot depend on a low interfacial tension between the phases. The considerable amount of work needed to produce a primary emulsion confirms the interfacial tension to be high yet in the time taken to make such an emulsion a tenacious film will form around the oil globule. On standing this film will increase in thickness and behave as an elastic solid thus accounting for the great stability of acacia emulsions.

References

1. Shotton, *J. Pharm. Pharmacol.*, 1955, **7**, 990.
2. Internation Critical Tables, 1928, IV, 436.
3. Porter, *Phil. Mag.*, 1933, **15**, 163.
4. Schulman and Cockbain, *Trans. Farad. Soc.*, 1940, **36**, pt. 6, 651.
5. Banerji, *Proc. Nat. Acad. Sci. India*, 1936, **6**, 317.
6. Clark and Mann, *J. biol. Chem.*, 1922, **52**, 157.
7. Matthews, *Trans. Farad. Soc.*, 1939, **35**, 1116.
8. Marshall, *Pharm. J.*, 1909, **82**, 257.
9. Bancroft, *J. Phys. Chem.*, 1913, **17**, 514.
10. Sellerach and Jones, *Industr. Engng Chem.*, 1931, **23**, 1017.
11. Sellerach, Jones and Owen, *ibid.*, 1933, **25**, 816.

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

THE SIZE ANALYSIS OF PHENOTHIAZINE

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Results are given for the analysis of particle size distribution of a sample of finely ground phenothiazine by four different methods. Each method is reproducible and is in use for the size analysis of phenothiazine in different laboratories, but the results differ considerably. The advantages and disadvantages of each method are discussed briefly, and a means of comparing the results with those obtained by surface area measurements is shown.

Phenothiazine, or thiodiphenylamine, has been used for many years as an anthelmintic, mainly in sheep and cattle. It is manufactured by the reaction of diphenylamine and sulphur in the liquid state, and crystallises from the melt on cooling, and one or more grinding processes have to be gone through before the material is of a suitable size for use.

Phenothiazine is practically insoluble in water and is now used either as a tablet, a dispersible powder, or a ready-made liquid suspension. The dispersible powder is the most popular in this country, and is included, together with the tablets, in the B.Vet.C. The particle size requirements of the British Veterinary Codex monograph are "not more than 0·1 per cent should be retained on a No. 25 sieve (600 microns) and not more than 5·0 per cent on the No. 100 sieve (150 microns)".

In 1956 Gordon[1] published results of experiments on sheep which showed that the finer the particle size the greater the anthelmintic effect, and since that time the emphasis on "fine particle size" phenothiazine has gradually increased. The particles in "fine particle size" phenothiazine are too small for a sieve analysis to be of value because the finest sieve which is robust enough for practical use is the 200 mesh B.S.S. which passes particles less than 76 microns. Size analysis of particles less than 76 microns and greater than 2 microns can be done by various means but a comparison between the results obtained on a single sample of phenothiazine has not so far been published.

Methods of Size Analysis

The two common methods of sizing in the "fine particle size" range are microscope counting and sedimentation. The results for the two methods are not identical since the parameters are different. With the microscope the projected area of the particle is matched with one of a series of circles on a reference graticule. In sedimentation methods the property measured is the free falling speed, and the particle size is defined as the diameter of a hypothetical sphere whose falling speed is the same as that of the particle under identical conditions. There are several variations of the sedimentation technique, three of which are described. These are the Andreasen pipette[2] (a fixed position pipette method), the Stairmand[3] apparatus (a

liquid column method with sediment extraction), and the Micromerograph[4] (a gas column method with sediment accumulation).

A third method, that of surface area measurement, which may be translated into mean particle size if desired, gives no information on the distribution of particle sizes in the sample. Two well-known methods of measuring surface area of solids are available: one involves measuring the volume of gas, usually nitrogen, adsorbed by a given weight of sample at liquid air temperatures[5], and the other the permeability of a compressed plug of powder, to a fluid, usually air, under ordinary atmospheric conditions[6]. The latter is simpler and gives reproducible results, but the results differ widely from those obtained by the former unless extensive corrections are applied.

Microscope Method

Phenothiazine is best prepared for microscope counting by suspending in arachis oil, and a suitable concentration is obtained by trial and error.

One drop of the suspension is placed on a microscope slide and a cover slip pressed lightly over it. The slide is examined with either a bench microscope, using an 8 mm. objective and a 10x eyepiece, or, preferably, a projection microscope. A reference graticule consisting of a series of circles and rectangles[7] is used. The diameter of the circles against which the particles are matched, are arranged as multiples of $\sqrt{2}$ starting with 76 μ as the highest value, and the rectangles, the lengths of whose sides are simple multiples of the circle diameters, form the fields, inside which the particles are counted. This means that successive circles each have twice the area of the preceding one. The number of fields counted depends on

TABLE I

MICROSCOPE SIZE ANALYSIS OF FINELY GROUND PHENOTHIAZINE

Size range (μ)		Volume factor $\frac{d_1^3 + d_2^3}{2}$	Number of fields observed	Number particles sized	Number of particles per basic area (n)	Volume per unit area nd^3	Weight per cent in grade	Weight per cent less than upper limit of size range
d_1	d_2							
26·5	18·8	12,627	20	14	14	176,778	15·15	100·00
18·8	13·2	4,472	20	48	48	214,656	18·39	84·85
13·2	9·4	1,565	20	124	124	194,060	16·63	66·46
9·4	6·6	559	10	188	376	210,184	18·01	49·83
6·6	4·7	196	5	208	832	163,072	13·97	31·82
4·7	3·3	69·9	5	364	1,456	101,774	8·72	17·85
3·3	2·3	24·1	2	204	3,040	73,264	6·28	9·13
2·3	0	6·1	2	546	5,460	33,306	2·85	2·85
						1,167,094		

the density of particles on the slide, and since the smallest particles greatly outnumber the largest they need not be counted in all the fields required to obtain a reasonable number of the largest particles. The fields to be counted are selected according to a definite scheme such as that given in Appendix XIII of the British Pharmacopoeia, 1958. Obviously the figures obtained, if used directly, give an analysis relating to the number of particles in given size ranges. It is usual to translate such figures into a weight analysis by assuming all the particles to be spheres of equal density.

The figures then correspond to those obtained by sedimentation methods. The data and derived information is collected in the form of a table (see Table I), and by plotting the figures for "Weight per cent less than upper limit of size range" in the last column against the upper limit of size range in the first column a cumulative curve representing the size distribution is obtained, from which the weight percentage less than any given size may be read.

Sedimentation Methods

These methods depend upon the calculation of free-falling speeds, by means of Stokes' Law, of particles in different size ranges or fractions, and give analyses in terms of weight of powder in a given fraction directly. Stokes' Law may be written,

$$t_1 = (18 \times 10^8 nh)/\{(p - p_1)\, g\, d_1^2\} \quad .. \quad .. \quad .. \quad (1)$$

where t_1 is the settling time for particles, of diameter $d_1\,\mu$ and density p, to fall a height h under the influence of gravity, g, in a fluid of viscosity n and density p_1.

In practice, unless a sedimentation balance, such as the Micromerograph, is used d_1 refers not to a particle of a single definite size, but to a size range, and according to the method, may be either the top size or the mean size of a fraction. As in microscope sizing, a $\sqrt{2}$ progression of diameters is used to define successive fractions and since the time of fall is proportional to the square of the diameter this means that each settling time is double the previous one, so that Stokes' Law has to be applied only once to determine the time, t_1, to extract the initial sample.

Andreasen Pipette

The apparatus consists of a glass parallel-sided sedimentation vessel with a ground glass neck, having a graduated scale 20–0 cm. marked on the side with the zero mark about 3 cm. from the bottom. The pipette is fitted with a two-way tap and side discharge tube and has a capacity of 10 ml. It has a ground glass socket below the bulb which fits into the neck of the sedimentation vessel. The stem from the pipette bulb to the sampling inlet, which coincides with the zero mark on the sedimentation vessel when the apparatus is in use, is made of fine capillary tubing.

A 5 g. sample of phenothiazine is dispersed in a suitable quantity of a solution of a wetting agent and transferred to the sedimentation vessel. It is further diluted to the 20 cm. mark (about 550 ml. in all) with the solution of the wetting agent, *not* water, mixed by inverting the vessel several times, and allowed to settle. The limiting Stokes' diameter, d_1, for the first fraction is taken as 76 μ, that is, all particles greater than 76 μ will have had time to fall 20 cm. and none will be collected, and the time, t, to extract the first sample is obtained from equation 1. Times to extract subsequent samples are all twice the previous time interval, except that a slight correction is made for the decrease in height of the column of liquid in the sedimentation vessel as samples are removed. The 10 ml. fractions are filtered through a No. 4 sintered glass crucible, vacuum dried over P_2O_5, and weighed. Each fraction then contains a mixture of

particles all less than a given diameter in the same proportion of sizes as was originally present in the complete sample, except that the oversize has been removed completely. The calculation required to obtain the weight percentage of particles less than the upper limit of a given size range is

$$\text{Weight per cent} = 100\ w/\mathrm{W}.\ \mathrm{V}/v.$$

where w = weight of fraction in g.
W = weight of initial sample in g.
V = volume of sedimentation vessel
v = volume of pipette

Table II gives the size analysis of the phenothiazine sample obtained with the Andreasen pipette. From this it can be seen that one very practical disadvantage of aqueous sedimentation methods is that in a

TABLE II

ANDREASEN PIPETTE SIZE ANALYSIS OF FINELY GROUND PHENOTHIAZINE

Size range (μ)	Time			Weight of sample (g.)	Weight per cent less than upper limit of size range
	Hr.	Min.	Sec.		
76–53	—	4	11	0·0940	100·6
53–37·5	—	8	10	0·0918	98·3
37·5–26·5	—	15	51	0·0914	97·8
26·5–18·8	—	31	0	0·0899	96·2
18·8–13·2	1	0	20	0·0874	93·5
13·2–9·4	1	57	20	0·0805	86·1
9·4–6·6	3	44	20	0·0742	79·4
< 6·6	7	13	—	0·0610	65·3

normal working day particle sizes down to 6·6 μ only can be collected. Since according to the results quoted, 65 per cent by weight of the powder under test is smaller than 6·6 μ the analysis can hardly be said to be completed, although sufficient information has been obtained for most purposes.

For the sedimentation vessel used $\mathrm{V} = 535$ ml., $v = 10$ ml., and $\mathrm{W} = 5{\cdot}0$ g., $\therefore$ Weight per cent $= 100\ w/5{\cdot}0 \times 535/10 = 1070\ w$.

Stairmand Method

This differs from the Andreasen pipette method in that the whole of the sedimenting solid is collected in the form of fractions removed at predetermined times, by allowing the sediment to flow out at the bottom of the glass sedimentation tube, which in our case is 32 cm. long. The volume of liquid and sediment removed is replaced by fresh solution from the reservoir. The limiting Stokes' diameter, d_1, is taken to be the geometric mean of the size limits and not the upper size limit, since the whole of this fraction together with varying proportions of the other fractions is collected after the first time interval t_1. Because of this the calculation is different to that used in the Andreasen pipette method, but is fully described in Stairmand's original paper[3]. In effect the weight obtained as fraction 1 is doubled and the weight of fraction 2 subtracted from it to obtain the weight of particles in the first size grade. This procedure is followed for each grade until the last fraction is reached when the weight of this is doubled and the weight of solids still remaining in suspension (obtained

by difference from the total weight originally put in to the apparatus) added to it. The results given in Table III show how this works out in practice, and the figures for "weight per cent less than mean size" in the last column can be compared with the figures obtained by the two previous methods.

Micromerograph

The particles of dry powder are sedimented in still air down a seven foot long vertical tube and collected on the pan of an automatic servo-controlled torsion balance at the bottom of the tube. The powder is dispersed at the beginning of the experiment by blowing it into the sedimentation column through an annular slit with a known volume of dry nitrogen. Because the adhesive force between particles, and their fragility,

TABLE III

STAIRMAND SEDIMENTATION SIZE ANALYSIS OF FINELY GROUND PHENOTHIAZINE

Size range (μ)	Mean sizes	Time			Weight of sample (g.)	Weight in grade	Weight per cent in grade	Weight per cent less than mean size
		Hr.	Min.	Sec.				
104–76	89	—	3	24	0·0223	0·0102	2·04	100·00
76–53	63	—	6	48	0·0121	0·0091	1·82	97·96
53–37·5	44·5	—	13	36	0·0151	0·0106	2·12	96·14
37·5–26·5 ..	31·5	—	27	12	0·0196	0·0119	2·38	94·02
26·5–18·8 ..	22·2	—	54	24	0·0273	0·0065	1·30	91·64
18·8–13·2 ..	15·7	1	48	48	0·0481	0·0294	5·88	90·34
13·2–9·4 ..	11·1	3	37	36	0·0668	0·0708	14·16	84·46
9·4–6·6	7·9	7	15	12	0·0628	0·0733	14·66	70·30
6·6–4·7	5·6	14	30	24	0·0523	0·0707	14·14	55·64
< 4·7	3·9	29	0	48	0·0341	0·2075	41·50	41·50

varies for different powders, a wide range of shear forces can be applied by varying the nitrogen pressure and the slit width, so that the optimum dispersion conditions can be found by trial and error methods for each material.

Since the powder accumulates steadily on the balance pan the calculation is not based on the time required to extract the first sample and the equation for Stokes' Law is rearranged so that particle diameter is given as a function of settling time: $d_1^2 = (18 \times 10^8 nh)/(p - p_1)\, g\, t_1$.

The density of the fluid p_1 is so small for air that it can be neglected; the viscocity of air, n, the height of fall, h, and the acceleration due to gravity, g, are all constant so that the equation may be written, $d_1 p^{\frac{1}{2}} = \mathrm{K} t_1^{\frac{1}{2}}$.

The method requires that the total duration of the run shall be predetermined. To calculate this it is assumed that a value of $d_1 p^{\frac{1}{2}} = 2$ represents the lowest size which will have any appreciable effect on the analysis, and this enables the entire run to be completed in about $3\frac{1}{2}$ hours. After the preliminary calculations have been made the Micromerograph produces automatically a chart representing the total weight of powder collected on the balance pan as a continuous function of time and from this the analysis is derived directly by means of a template supplied by the makers. The experimental values are given in Table IV.

Surface Area Methods

The surface area of a powder can be measured by various means but the one in common use, owing to its simplicity, is air permeability, and the apparatus available can be divided into two classes.

The first one depends on a direct measurement of the pressure drop across a compressed plug of powder when air at a fixed inlet pressure is passed through it. This was developed by Carman[6] and is now sold commercially as the "Fisher Sub-Sieve Sizer". The apparatus includes a complicated chart from which a mean particle size is read directly, and

TABLE IV

MICROMEROGRAPH SIZE ANALYSIS OF FINELY GROUND PHENOTHIAZINE

Pressure 150 psi. Slit width 150 μ

$dp^{\frac{1}{2}}$	$d(\mu)$	Recorder chart reading	Weight per cent less than d
66	56·5	0	100
60	51·5	0·2	99·3
54	45·3	0·2	99·3
48	41·2	0·2	99·3
42	36·0	0·3	99·0
36	30·9	0·3	99·0
30	25·8	0·3	99·0
27	23·2	0·4	98·7
24	20·6	0·6	98·0
21	18·0	1·5	95·1
20	17·2	1·9	93·8
19	16·3	2·3	92·5
18	15·5	3·0	90·2
17	14·6	3·9	87·2
16	13·7	5·0	83·6
15·1	13·0	6·0	80·3
14	12·0	7·8	74·4
12	10·3	11·9	61·0
10	8·6	16·2	46·9
8	6·9	20·7	32·2
6	5·2	25·7	17·0
5	4·3	27·3	10·7
4	3·4	29·0	5·0
3	2·6	30·0	1·8
2·5	2·1	30·3	0·8
2	1·7	30·6	0

results are, therefore, obtained in terms of a mean particle size derived from what is actually a surface area measurement. The implications of this are discussed later.

The second variation depends on the time taken for oil in a manometer tube to approach equilibrium by flowing under gravity between two fixed levels, the air so displaced being forced through a compressed plug of the powder. This was developed by Rigden[8].

The basic equation for the calculation of specific surface which applies to both variations, was evolved by Kozeny from Poiseuille's Law, and may be written, $S^2 = \frac{A}{knLp^2} \cdot \frac{e^3}{(1-e)^2} \cdot \frac{\Delta P}{V}$.

where S = specific surface area in cm.^{2}/g. A = area of cross section of the plug of powder in cm.^{2} L = length of plug of powder in cm. n = viscosity of air = $1{\cdot}81 \times 10^{-4}$ poises at room temperature. p = density of powder = 1·36 for phenothiazine. k = Kozeny's constant = 5·0. e = Porosity factor or void volume = volume of air in 1 g. of compressed

powder. ΔP = pressure drop across the plug, and V = velocity of air through the plug in cm./sec.

The porosity factor is defined as $e = (AL - W/P)/AL$ where W is the weight of sample in g. The calculation may be simplified if the length of the plug, L, is kept constant and the weight of sample is numerically equal to the density of the sample. This is the recommended method of using the Fisher Sub-Sieve Sizer, since the only variables are then ΔP and V which are readily measured by means of a water flowmeter. Rigden prefers the plug of powder to be packed using as far as possible the same pressure, so that L and e vary with each sample. This makes the calculation slightly more lengthy but is more accurate. The calculation of $\Delta P/V$ is mathematically more difficult with the Rigden apparatus since the air pressure drops steadily throughout the experiment, but this is largely an instrumental factor and once it has been determined the only experimental data required is the time taken for oil to flow between two fixed points in a manometer.

The results on the sample of phenothiazine are as follows:

Fisher Sub-Sieve Sizer:	Mean size 2·4 μ
	Equivalent to a surface area of 18,400 cm.^{2}/g.
Rigden Apparatus:	Surface area 14,400 cm.^{2}/g.

There is a fixed difference between these two pieces of apparatus, the Fisher giving results about 25 per cent higher than the Rigden, which is probably caused by inaccuracies in determining the instrumental constants. Since it is not known which is the more correct no attempt has been made to alter the constants of either apparatus.

Discussion

The methods described are all in use throughout the world for the size analysis of phenothiazine, but where results are quoted in the manufacturers' literature insufficient attention is paid to the differences which arise from the use of different methods. The magnitude of these differences can be seen if figures for the percentages of particles less than 30 μ and less than 10 μ, obtained by the different methods on the same sample of phenothiazine are taken from Figure 1, as follows.

	$<30\,\mu$	$<10\,\mu$
Microscope	98·4	55 per cent
Andreasen pipette	97·4	80 per cent
Stairmand	97·4	78 per cent
Micromerograph	98·8	60 per cent

Each of these methods give reproducible results, but it is practically impossible to determine which, if any, of them is correct. Obviously, if every manufacturer of phenothiazine used the same method of analysis this would not be necessary, but since, so far, they do not, it is desirable to have some idea of the advantages and disadvantages of each method.

The two wet sedimentation methods require very little special apparatus beyond that normally present in a chemical laboratory and are, therefore, popular on the grounds of economy. They are, however, the most time-consuming and probably the least accurate since flocculation of the finest particles occurs if the settling time is prolonged, and Brownian movement of particles less than 2 μ completely upsets the Stokes' Law relationship. The turbulence which exists immediately after the contents of the tube have been mixed makes the zero time difficult to interpret and can cause errors in the estimation of the first few samples. Inspection of Figure 1 shows that the results obtained in the highest fractions do not

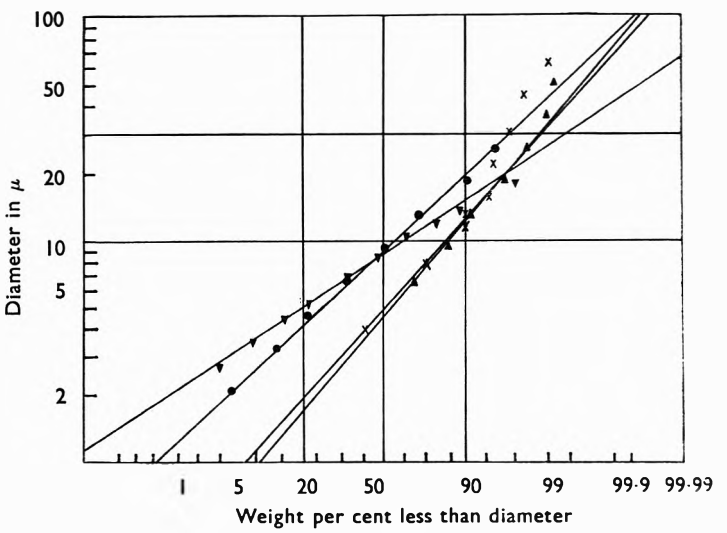

FIG. 1. Logarithmic probability graph of the particle size distributions of a phenothiazine sample by four different methods.

▼—▼ Micromerograph. ×—× Stairmand.
●—● Microscope. ▲—▲ Andreasen.

fit the lines drawn to represent the distributions. Apart from such inaccuracies the time factor is important because a complete experiment cannot be fitted into a normal working day if sizes less than 6 to 7 μ require estimation. The results obtained by these two methods are similar, as would be expected, the chief difference being that since the Stairmand apparatus requires more dilute suspensions the particles should obey Stokes' Law more closely and therefore a more accurate result should be obtained. On the other hand, the Andreasen pipette is the only method of those considered where only one sample needs to be taken at a pre-determined time to produce a percentage figure less than any required size, without the necessity for determining a complete analysis. This simplified treatment makes the method much more attractive since it eliminates the inaccuracies at both ends of the distribution and yields all the information required for routine control, if two samples, corresponding to sizes less than, say, 30 μ and 10 μ are taken.

The microscope counting method is perhaps the most well known of all methods of sizing particles, but is often avoided as being very tedious for routine measurement and liable to produce eye-strain in the operator. Using a projection microscope and an experienced operator, however, complete analyses can be produced with a high degree of reproducibility within two hours. Where results are required in a hurry this is an obvious advantage over wet sedimentation methods. The statistical necessity of obtaining a reasonable number of the largest particles when counting places a restriction on the top limit of particles sized and it may be that

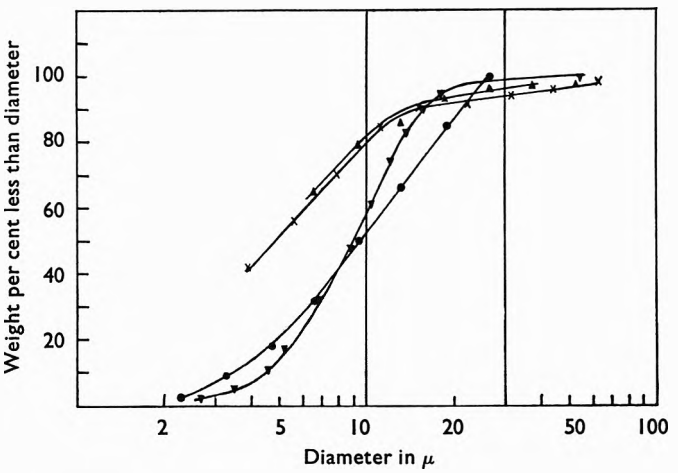

FIG. 2. Cumulative plot of the particle size distributions of a phenothiazine sample by four different methods.

▼—▼ Micromerograph. ×—× Stairmand.
●—● Microscope. ▲—▲ Andreasen.

one or two of the particles included in the top grade are bigger than the upper limit. For this reason the size corresponding to 100 per cent less than the upper limit of size range is always smaller than that obtained by sedimentation methods (see Fig. 2). In producing a weight analysis from a count of the number of particles it is usually assumed that all particles are spherical. The larger particles of phenothiazine may, however, deviate considerably from sphericity since the crystals are flaky, which means that the large particle end of a distribution becomes over-emphasised so that in this region a microscope analysis gives higher results than other methods. Stokes' Law as applied to sedimentation methods also assumes that the particles are spherical but small deviations from sphericity have a negligible effect on free-falling speed.

The Micromerograph is a very expensive piece of apparatus and one not likely to be bought by anyone whose sizing problems are with phenothiazine only, unless his manufacture is on a very large scale. Once installed, however, it is a useful instrument which gives reproducible results with a minimum of attention and within a few hours. It is the only one of the four instruments considered, whose use is restricted to dry

powders, so that ready-made aqueous suspensions or drenches cannot be analysed. There is some doubt whether complete dispersion without grinding is achieved; certainly results on phenothiazine always show a closer-sized distribution than any other method which could mean that some grinding of the large particles occurs when the material is blown into the sedimentation chamber whilst the smallest particles are not completely dispersed and fall as small aggregates. The agreement between the microscope and Micromerograph analysis in the middle ranges of the distribution is reasonably good.

Surface area measurements can be compared only with particle size distributions if some assumptions are made about the type of distribution usually encountered. It is generally accepted[9] that size analyses of ground powders, where the material is the product of a single grinding operation and has not been obtained by mixing products ground to different degrees, obey at least approximately a logarithmic probability law. For this reason one of the most popular ways of plotting the results of a size analysis is as a cumulative graph of weight per cent less than a given diameter on an arithmetic co-ordinate, against the given diameter on a logarithmic co-ordinate. This gives a symmetrical S-shaped curve, and Figure 2 shows the results quoted in Tables I to IV plotted in this way. Particle size requirements for phenothiazine are usually requested as a certain percentage less than a given size and from this graph such information can be read.

The statistical equation for a logarithmic normal distribution may be written, $df/\mathrm{N} = 1/\sqrt{2\pi} ln\,\alpha \exp.\,[-(ln\mathrm{D}-ln\mathrm{M})^2/2ln^2\,\alpha]\,\alpha ln\mathrm{D}$, when f is the frequency with which a particle of diameter D occurs in a number of particles, N. M is the geometric mean diameter and α the geometric standard deviation of the distribution. The equation relates the fractional numbers of particles in each size grade (f/N) to the logarithm of the diameter of the size grade (lnD) and represents a non-cumulative distribution completely defined in terms of two parameters M and α. The standard deviation, α, and the weight mean diameter, d_w, are obtainable by the application of the above equation to a plot of the results of particle size analyses on logarithmic probability paper (see Fig. 1) and it only remains to relate d_w with the surface mean diameter d_s to calculate the surface area figure.

Referring to Figure 1 the weight mean diameter is the diameter corresponding to the 50 per cent figure on the probability co-ordinate, and the standard deviation is the diameter corresponding to the 84·13 per cent figure divided by the 50 per cent figure. These two parameters can thus be determined directly from the graph for all four size distributions. To use surface area measurements a surface mean diameter, d_s, must be defined. This can be done by means of two of the Hatch-Choate[10] equations, $\log d_s = \log \mathrm{M} + 5{\cdot}757 \log^2 \alpha$, and $\log d_w = \log \mathrm{M} + 8{\cdot}059 \log^2 \alpha$; log M, whose value is unknown, can be eliminated from these two equations, and $\log d_s$ related directly to low d_w, as $\log d_s = 2 \log d_w - 5{\cdot}302 \log^2 \alpha$. If it is assumed that the particles are spheres then the relationship between the surface mean diameter, in microns, and the

specific surface area in cm.²/g. is given by, $S_w = 6 \times 10^4/p.d_s$ where p is the density of phenothiazine (1·36 g./cm.³).

The derived values for the standard deviation, the weight mean diameter, the surface mean diameter, and the surface area of the four size distributions are given in Table V, together with the air permeability values, obtained from the Sub-Sieve Sizer and the Rigden apparatus, for surface area and surface mean diameter.

It must be realised that the data for the Stairmand and Andreasen sedimentation methods is incomplete and the extrapolation required to produce the straight line on the logarithmic probability graph may have introduced serious errors.

This argument shows how a size analysis, provided it follows the log probability law may be equated to a surface area value but it must be realised that the converse does not hold. A surface area measurement

TABLE V

SURFACE AREA VALUES FOR FINELY GROUND PHENOTHIAZINE

Method	$d_w(\mu)$	α	$d_s(\mu)$	S_{ic} (cm²/g.)
Micromerograph	8·75	1·72	6·52	6,800
Microscope	9·2	2·17	5·05	8,750
Stairmand	4·9	2·45	2·20	20,100
Andreasen	4·6	2·62	1·82	24,200
Sub-Sieve Sizer	—	—	2·4	18,400
Rigden	—	—	3·3	13,400

can relate to any number of distributions and therefore such a measurement cannot be used to define a distribution completely. Moreover it can be applied only to dry powders and the method, like the Micromerograph method, cannot be applied to suspensions in water. Another disadvantage is that the effect of wetting agents which are present in dispersible powders, even in quantities as low as 0·5 per cent, materially affect the surface area figures obtained and comparison of samples of phenothiazine dispersible powder whose origin and formulation is unknown, cannot be attempted. The only worth-while application of surface area measurements is, therefore, as a routine check of the grinding efficiency of a mill, for which purpose they are ideally suited.

Some attempts have been made, both in this country and in South Africa and New Zealand, to define a method for the standardisation of particle size analysis of phenothiazine. The generally preferred method, mainly on the grounds of cheapness and availability of apparatus than on inherent accuracy, is the Andreasen pipette.

Acknowledgement. I am indebted to Mr. M. W. Vincent of Sharples Super Centrifuges Limited, for the Micromerograph analysis.

REFERENCES

1. Gordon, *Amer. J. Vet. Res.*, 1956.
2. Andreasen, *Kolloidbeihefte*, 1928, **27**, 349.
3. Stairmand, *Trans. Inst. Chem. Eng.*, 1947, **25**, 128.
4. Eadie and Payne, *Iron Age*, 1954, **10**, 99.
5. Brunauer, Emmett and Teller, *J. Amer. chem. Soc.*, 1938, **60**, 309.
6. Carman, *J. Soc. Chem. Ind.*, 1939, **58**, 1.

7. Fairs, *Chem. Ind.*, 1943, **62**, 375.
8. Rigden, *J. Soc. Chem. Ind.*, 1943, **62**, 1.
9. Dallavalle and Orr, *Fine Particle Measurement*, Macmillan, London.
10. Hatch and Choate, *J. Franklin Inst.*, 1929, **207**, 369.

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

All the methods referred to by the author were reliable. The Andreasen pipette was probably the most widely used. The author preferred the microscopical method, but the use of a haemocytometer was not satisfactory, as the counting chamber was too deep. Self-attrition, a problem encountered with a centrifugal classifier, was also a failing of the micromerograph. Anomalous results with blended samples of phenothiazine had not been encountered by the author. Flocculation sometimes caused difficulties in wet sedimentation methods, and the choice of wetting agent was important.

FURTHER STUDIES ON SOME ESTERS OF 4-AMINOSALICYCLIC ACID

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A series of alkyl and aryl esters of 4-aminosalicyclic acid (PAS) has been examined for protective effect in mice infected with *Mycobacterium tuberculosis* H37Rv, and the PAS blood levels after oral administration to mice, rats and rabbits studied. On the basis of these results, *p*-tolyl-4-aminosalicylate (*p*-tolyl PAS) was selected for further examination and has been shown to be of low toxicity in experimental animals and to produce moderately high and sustained PAS blood levels in man.

The widespread use of 4-aminosalicylic acid (PAS) or its sodium salt as an auxiliary chemotherapeutic agent in the treatment of tuberculosis has directed much effort towards rendering this drug more palatable and less irritating to the gastrointestinal tract. A wide variety of pharmaceutical formulations such as tablets, granules and cachets have appeared which overcome the taste of PAS, but do little to mitigate its other side effects.

Calcium 4-benzamidosalicylate, a tasteless derivative of PAS which is hydrolysed in the body to PAS, has been shown[1,2] to be better tolerated than PAS and has recently become widely used as a substitute. However, the lower PAS blood levels produced with this substance led us to re-examine a series of esters of PAS, the tuberculostatic activity of which have been previously described[3] and further studies on these compounds are now reported.

Experimental Methods

Antituberculosis activity. *In vivo* tests for tuberculostatic activity were carried out by the following method. Mice (18–20 g.) were injected intravenously with 0·3 ml. of a 10-day culture of *Mycobacterium tuberculosis* H37Rv and divided into groups of 10. One group served as an untreated control, and a second group received a diet containing 0·5 per cent sodium PAS. Other groups were given the test compounds suitably diluted in the diet. Median survival times (ST50) and 95 per cent confidence limits were determined using the graphical method of Litchfield[4] and chemotherapeutic activity expressed as the difference between the ST50s of treated and control animals (ΔST50).

PAS blood levels in rabbits. Drugs were given by stomach tube to rabbits, using two animals for each compound. Blood was taken 1, 2, 4 and 6 hours after drug administration.

PAS blood levels in mice. Drugs were given by stomach tube to groups of mice. Six animals were killed from each group at 1, 2, 4 and 6 hours after drug administration, and the pooled blood used for estimation of PAS and esterified PAS.

PAS blood levels in man. Drugs were given orally to healthy male volunteers, using three volunteers for each compound. Blood was taken 1, 2, 4 and 6 hours after drug administration.

PAS blood levels during conditions of a mouse survival test. Groups of mice were fed diets containing sodium PAS, phenyl PAS or *p*-tolyl PAS, at a dose equivalent to 0·5 per cent sodium PAS in the diet, for 14 days after which three animals from each group were killed at 2-hourly intervals over a 24-hour period, and the pooled blood taken for determination of PAS.

Tissue PAS levels in rats. Rats were pre-treated for 14 days with sodium PAS or *p*-tolyl PAS with a dose equivalent to 1 per cent sodium PAS in the diet. They were starved on the fourteenth night and on the following morning received a single dose of the appropriate drug equivalent to 500 mg./kg. sodium PAS, by stomach tube. Blood and tissue samples were taken 1 hour and 3 hours after drug administration. A control series of animals, which had not received the 14 days' pre-feeding, was included.

Blood PAS levels in humans after repeated drug administration. Twelve male volunteers were divided into three groups of 4, and treated for 15 days with sodium PAS, phenyl PAS, or *p*-tolyl PAS in three daily doses, using doses of drugs stoichiometrically equivalent to 3×60 mg./kg./day of sodium PAS. Blood samples were taken five times at 3-hourly intervals during the first and last days of treatment, and once on days 5, 9 and 12, and assayed for PAS content.

Total PAS. Estimations of total PAS, acid plus ester, were made by the method of Newhouse and Klyne[5], with the modification that diazotisations were at 0° to minimise errors due to small departures from the time schedule.

Esters of PAS in blood. The method described by Frederiksen, Jensen, Mørch, and Tybring[6] for the estimation of phenyl-4-aminosalicylate and phenyl PAS, in blood was not applicable to all the esters, and the following method was used. 2 ml. of blood was added to 6 ml. of 0·05N sodium hydroxide in a stoppered boiling tube. Ether, 30 ml., was added and the tube shaken for 10 minutes and the contents allowed to settle. 25 ml. of the ether layer was transferred to a 200 mm. B.24 stoppered boiling tube and evaporated to dryness in a stream of air. Water, 5 ml., and concentrated hydrochloric acid, 1·5 ml., were added, and after shaking to dissolve material adhering to the sides of the tube, diazotisation and coupling were carried out by the Newhouse and Klyne technique. After standing for 30 minutes, ethanol, 5 ml., was added and the colour density measured at 555 mμ. Standards were prepared by dissolving the ester, 8 mg., in ethanol, 100 ml. To 0·5 ml. of this solution was added 6 ml. 0·05N sodium hydroxide and 2 ml. normal blood, and the above procedure followed. Blank determinations were made using 2 ml. normal blood.

PAS in tissues. Animals were killed by decapitation and the liver and kidneys were freed from blood by perfusion with saline at 37°. This required about 50 ml. of saline. Lungs were similarly treated by perfusion into the vena cava and the heart. The organs after weighing were

homogenised with water to give a final concentration of 1 g. of tissue in 20 ml. of homogenate. 2·4 ml. of homogenate was shaken with 2 ml. of 10 per cent trichloroacetic acid and water, 4·5 ml., added. After 5 minutes, the mixture was filtered and the filtrate treated by the Newhouse and Klyne technique.

Total PAS derivatives in urine. Total urine excreted over 24 hours was collected into Winchesters containing a little toluene as preservative. The volume was measured and total PAS derivatives estimated after

TABLE I

INCREASE IN MEDIAN SURVIVAL TIME (△ST50) IN MICE INFECTED WITH *M. TUBERCULOSIS* H 37RV

(95 per cent confidence limits in parentheses)

Compound	Dose	△ ST50 (days)
Sodium PAS	0·5 per cent in diet	+ 17 (10–24); + 13 (9–17); + 22 (14–32)
Phenyl PAS	≡ 0·5 per cent Na PAS in diet	+ 1 (0–4); + 18 (8–31); + 4 (0–8)
p-Tolyl PAS	,,	+ 15 (8–22); + 13 (9–17); + 19 (11–28); + 23 (17–29
o-Tolyl PAS	,,	+ 7 (1–13)
m-Tolyl PAS	,,	+ 6 (1–12); + 12 (5–19)
Ethyl PAS	,,	−3 (−8–+ 2); −3 (−8–+ 2)
β-Hydroxyethyl PAS ..	,,	+ 5 (0–10); + 7 (1–13)

TABLE II

PAS BLOOD LEVELS (μG./ML.) IN RABBITS FOLLOWING AN ORAL DOSE EQUIVALENT TO 500 MG./KG. Na PAS $2H_2O$

Compound	1 hr.		2 hr.		4 hr.		6 hr.	
	Total PAS	Ester	Total PAS	Ester	Total PAS	Ester	Total PAS	Ester
Sodium PAS	78 86	0 0	53 39	0 0	17 25	0 0	6 12	0 0
Phenyl PAS	6 5	1 2	9 2	0 1	14 7	1 1	11 6	1 1
p-Tolyl PAS	3 14	0 1	3 12	0 0	21 13	0 0	13 6	0 0
m-Tolyl PAS	6 10	3 1	5 11	0 0	7 22	1 0	6 19	2 0
o-Tolyl PAS	11 15	3 1	17 —	0 0	26 25	1 2	13 26	2 1
Ethyl PAS	41 26	1 1	45 16	1 0	29 13	0 0	20 5	0 0
β-Hydroxyethyl PAS ..	67 40	2 5	53 20	0 1	14 17	0 0	3 5	0 0

hydrolysis at 40° using the method of Way, Smith, Howie, Weiss and Swanson[7].

Chromatographic examination of urine. Urine was applied directly to Whatman No. 1 paper and developed with *n*-butanol : ethanol : 2N ammonium hydroxide, 4 : 1 : 5 by the ascending technique. Spots were detected either by dipping in Ehrlich's reagent for free amines, or by spraying with ferric nitrate solution.

Acute toxicities. Acute toxicity determinations were made in rats and mice, drugs being given orally by stomach tube.

Chronic toxicity of p-*tolyl PAS.* Forty male rats (70 to 100 g.) were divided into four equal groups. One group served as a control and the others received *p*-tolyl PAS, administered in the diet, at doses of 250, 625, and 1560 mg./kg. The animals were weighed twice weekly and growth curves plotted for each group. After 14 weeks, determinations of blood urea, glucose, and haemoglobin, red cell, white cell, differential white cell, and reticulocyte counts, urine albumen and glucose were

TABLE III

PAS BLOOD LEVELS (μG./ML.) IN MICE FOLLOWING AN ORAL DOSE EQUIVALENT TO 500 MG./KG. Na PAS $2H_2O$

Compound	1 hr.		3 hr.		5 hr.	
	Total PAS	Ester	Total PAS	Ester	Total PAS	Ester
Sodium PAS	63	4	0	0	0	0
Phenyl PAS	17	6	4	0	4	0
p-Tolyl PAS	14	2	2	1	6	1
Ethyl PAS	39	9	17	3	8	1
3-Hydroxyethyl PAS	132	36	17	1	2	2

TABLE IV

PAS BLOOD LEVELS* (μG./ML.) IN MAN AFTER AN ORAL DOSE EQUIVALENT TO 80 MG./KG. Na PAS $2H_2O$

Compound	1 hr.	2 hr.	4 hr.	7 hr.
Sodium PAS	45	55	17	3
	96	67	28	4
	80	48	12	0
Phenyl PAS	8	6	4	3
	19	11	5	3
	12	4	3	3
p-Tolyl PAS	19	11	2	0
	12	7	6	3
	25	14	5	0

* Blood levels of esterified PAS were also determined and found to be zero at all times.

carried out. After 17 weeks the animals were killed and specimens of liver, lung, heart, stomach, kidney, spleen, thyroid and adrenals taken for histological examination.

RESULTS

Chemotherapeutic tests. Table I shows the results of mouse survival tests on a series of PAS esters, compared with sodium PAS. It is apparent that *p*-tolyl-4-aminosalicylate (*p*-tolyl PAS)[8,9] displays consistently similar protection to that of sodium PAS. Other aryl esters, and the β-hydroxyethyl ester appeared to be considerably less active than sodium PAS, and the ethyl ester was inactive. This latter result is in agreement with previous reports of the lack of *in vivo* antituberculosis effect of alkyl esters of PAS. As it seemed likely that the protective effect of PAS esters was due to PAS produced by hydrolysis in the body, it was of

interest to determine whether the superior protective effect of *p*-tolyl PAS was due to PAS blood levels higher than those achieved with the other esters.

TABLE V

PAS BLOOD (μG./ML.) AND TISSUES (μG./G.) LEVELS IN RATS AFTER A SINGLE ORAL DOSE OF 500 MG./KG. Na PAS $2H_2O$ OR 576 MG./KG. *p*-TOLYL PAS, WITH AND WITHOUT 14 DAYS PRE-FEEDING AT A LEVEL OF 1·0 PER CENT IN DIET

Tissue	PAS levels in μg./ml. or μg./g.			
	1 hr. control	1 hr. pre-fed	3 hr. control	3 hr. pre-fed
	Sodium PAS			
Blood	167 174	152 156	23 12	100 57
Kidney	73 199	485 417	193 41	315 167
Liver	123 —	100 88	16 10	60 40
Lung	14 22	103 48	7 6	30 14
	p-Tolyl PAS			
Blood	19 14	51 51	7 4	51 30
Kidney	51 51	116 181	20 16	75 93
Liver	12 10	36 54	4 3	30 48
Lung	2 2	6 26	0 0	20 —

TABLE VI

PAS BLOOD LEVELS (μG./ML.) IN HUMAN VOLUNTEERS FOLLOWING ORAL ADMINISTRATION OF DOSES EQUIVALENT TO 3 × 60 MG./KG. Na PAS $2H_2O$/DAY FOR 15 DAYS

(Drugs administered at 08.00, 13.00 and 18.00 hours)

Subject	Drug	Day 1					Day 5	Day 9	Day 12	Day 15					Day 16
		09.00	12.00	15.00	18.00	21.00	15.00	15.00	15.00	09.00	12.00	15.00	18.00	21.00	09.00
M.W.	Sodium*	46	3	37	0	13	36	—	36	51	14	43	21	61	0
M.M.	PAS	49	8	22	0	14	—	43	—	48	19	42	10	27	0
K.T.		54	7	36	1	26	25	16	64	70	12	48	9	36	0
mean of the group		50	6	32	0	18				56	15	44	13	41	
R.R.†	Phenyl	27	4	11	1	19	18	—	22	—	—	—	—	—	—
G.V.B.	PAS	13	1	0	0	0	11	10	25	12	1	20	4	16	0
R.H.		13	4	11	3	11	8	30	32	14	14	27	11	24	0
L.M.		11	0	5	1	8	14	13	19	16	1	17	9	27	0
mean of the group		16	2	7	1	9				14	5	21	8	22	
F.J.	*p*-Tolyl	4	6	13	1	10	23	41	75	11	25	48	38	46	0
A.A.	PAS	12	7	22	3	9	57	32	52	24	17	29	10	14	0
W.S.		22	3	31	2	7	35	35	42	15	6	38	9	36	0
G.A.P.		17	2	5	3	8	26	6	5	16	19	16	29	43	5
mean of the group		14	4	18	2	8				16	17	33	21	35	

* One of the sodium PAS group left the trial on Day 2 due to illness.
† R.R. was withdrawn from the trial on Day 13 due to gastrointestinal side-effects of the drug.

Biochemical. Tables II, III and IV show the blood levels of both total PAS, acid and ester, and esterified PAS after oral administration of single doses of sodium PAS, and PAS esters to mice, rabbits, and humans. In all three species, sodium PAS is rapidly absorbed and excreted giving a

peak level approximately 1 hour after administration, the two alkyl esters show a similar pattern. Aryl esters give much lower levels in all the species, and in rabbits show a different pattern, the PAS levels rising gradually over the 6-hour observation period. With the exception of the

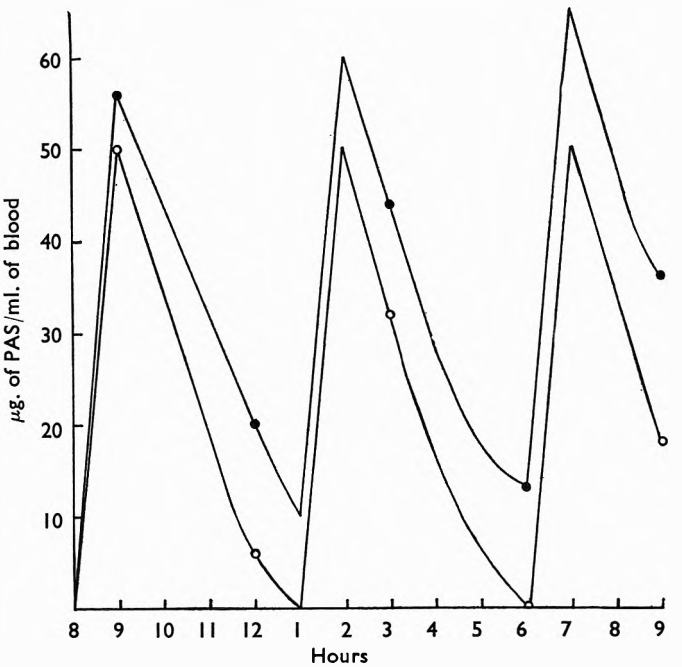


FIG. 1. Mean PAS blood levels (μg./ml.) of 3 volunteers receiving Sodium PAS. "Day 1" and "Day 15".
○ = Day 1. ● Day 15.

two alkyl esters in mice, the blood contains little or no esterified PAS, indicating rapid hydrolysis; in subsequent work, total PAS only was measured.

The blood PAS levels under conditions of mouse survival tests were very low, ranging from 0–6 μg./ml. Mean values for the three drugs examined were, sodium PAS, 3·0 μg./ml., phenyl PAS, 2·5 μg./ml., and *p*-tolyl PAS, 2·6 μg./ml.

Table V gives the PAS levels in blood, kidney, liver and lung of groups of rats following a single oral dose of sodium PAS or *p*-tolyl PAS, with and without 14 days pre-feeding of the appropriate drug. PAS levels were markedly higher in the pre-fed groups than in the control animals, and these results, together with the report of Torning[10] that blood PAS levels in patients treated daily with phenyl PAS steadily increased over a 3-week period, led us to investigate the PAS blood levels produced by some of these esters after repeated administration to man according to current clinical practice.

The results (Table VI) show that PAS blood levels were appreciably higher on day 15 than on day 1, and that the differences between mean

values for the 2 days were significant ($P<0{\cdot}05$), except for the 0900 hour readings where, in all three groups, the increases were not significant. By plotting mean values for day 15 and using suitable interpolated points,

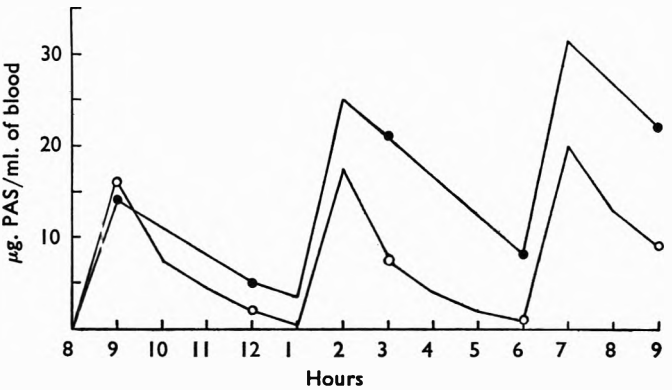


FIG. 2. Mean PAS blood levels (μg./ml.) of 4 volunteers receiving phenyl PAS. "Day 1" and "Day 15".
○ = Day 1. ● = Day 15.

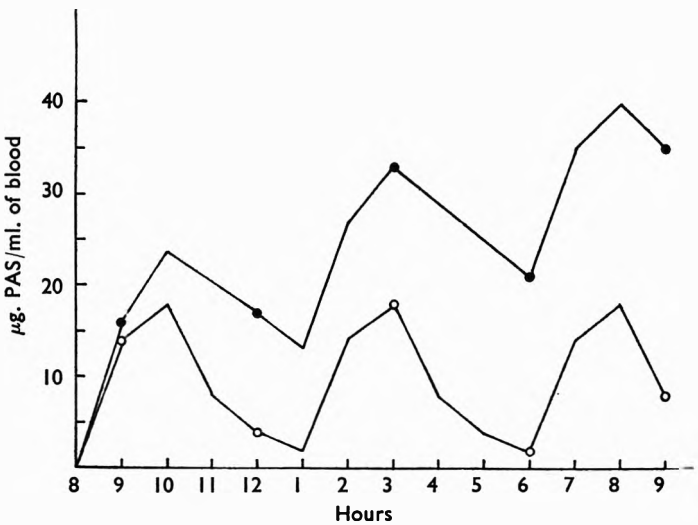


FIG. 3. Mean PAS blood levels (μg./ml.) of 4 volunteers receiving *p*-tolyl PAS. "Day 1" and "Day 15".
○ = Day 1. ● = Day 15.

an average blood level curve was constructed for each drug, these are shown in Figures 1, 2 and 3. By measurement of the area underneath the curves, an overall mean blood level for each drug during the observation period has been calculated, sodium PAS, 33 μg./ml., phenyl PAS, 16 μg./ml., and *p*-tolyl PAS, 24 μg./ml.

Twenty-four hour urine samples were collected from all subjects during the tenth day to determine the extent of absorption. Mean values for the

three groups, expressed as percentage of daily dose, were sodium PAS, 83 per cent (range 79 to 85 per cent), phenyl PAS, 63 per cent (range 43 to 74 per cent), and *p*-tolyl PAS, 68 per cent (range 61 to 88 per cent). Chromatographic examination revealed in all instances the presence of PAS, acetyl PAS and 4-aminosalicyluric acid, indicating a similar metabolic pattern for the PAS fragment of all three drugs.

Acute toxicity. Median lethal doses (oral) were in mice ethyl PAS, 1·5 g./kg., β-hydroxyethyl PAS, 2·0 g./kg., and *p*-tolyl PAS, about 10 g./kg. Mice receiving 1·0 g./kg. and above of β-hydroxyethyl PAS or ethyl PAS showed macroscopic pathological changes in liver, kidney and spleen.

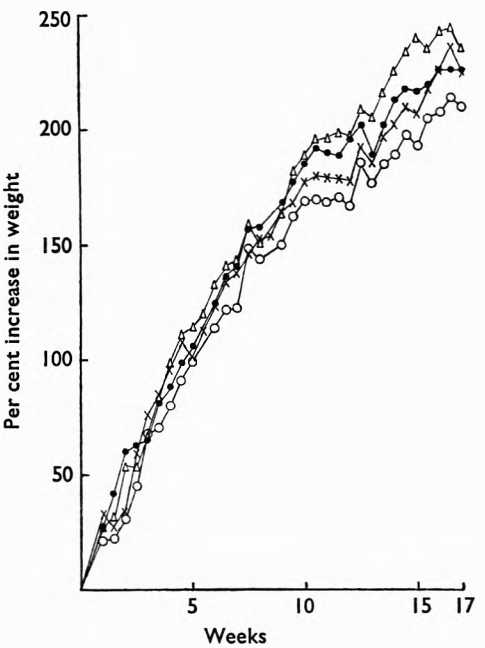


FIG. 4. The effect of *p*-tolyl PAS on growth of rats (10 animals per group).
○ = 1563 mg./kg. ● = Control
× = 625 mg./kg. △ = 250 mg./kg.

Ethyl PAS, and β-hydroxyethyl PAS in rats gave no deaths on doses up to 8·0 g./kg. orally, but all the animals except those receiving 1·0 g./kg. ethyl PAS showed pathological changes in liver, kidneys and spleen.

Chronic toxicity of p-*tolyl PAS.* Growth curves (Fig. 4) show that those animals receiving the highest dose, 1560 mg./kg., had a slightly reduced growth rate compared with the controls. Statistical analysis showed this decrease to be not significant. Blood urea and glucose, and urine albumen and glucose were within the normal range. Blood haemoglobin and red cell counts showed no difference from the controls when analysed statistically, but with the animals on the highest dose the white cell count (mean, 10,150/cu. mm.) was slightly less than the control value (mean, 13,300/cu. mm.), and the reticulocyte count (mean, 1·2 per cent) was slightly greater than the control value (mean, 0·5 per cent). Animals receiving the two lower doses showed no significant alteration in white cell or reticulocyte counts. A preliminary examination of sections from the animals receiving 1560 mg./kg. has shown no evidence of tissue damage, but the complete histological findings are not yet available.

DISCUSSION

Examination of the results of mouse survival tests (Table I) together with those of the chemically determined PAS blood levels (Tables II, III

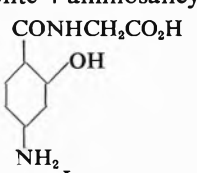
IV) shows some surprising features. Aryl esters of PAS give low PAS blood levels yet many show a marked protective effect on infected mice. Alkyl esters (ethyl and β-hydroxyethyl) give high PAS blood levels, but give less protection. It was thought that the first of these observations could be explained by postulating that, during the continuous dosage conditions of the mouse survival test, build up of PAS occurred, leading to higher blood levels after several days. The results indicate that this is not so, but do show the low PAS blood levels required for a protective effect in this test.

The low protective effect of ethyl and β-hydroxyethyl PAS confirms previous reports[3,11] on the inactivity of the alkyl esters of PAS *in vivo* and may be ascribed to toxic effects masking any protection afforded by the liberated PAS. Examination of mice receiving doses equivalent to those used in the mouse survival test (0·5 per cent in diet = about 1·25 g./kg.) showed pathological changes in liver, kidneys and spleen.

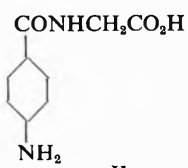
The effect of pre-feeding rats for 14 days with sodium PAS or *p*-tolyl PAS on the PAS blood and tissue levels after a single oral dose, was to increase considerably the PAS levels. The fact that the decline from 1-hour level to 3-hour level was much less in the pre-fed animals than in the controls may indicate some saturation of the enzymes responsible for the metabolic transformation of PAS, or maybe a block of excretion. The reason for the similarity in the 1-hour levels in the two sodium PAS groups may be that, with this drug, blood levels at 1 hour are controlled largely by its very rapid absorption rate, and any alteration in excretion rate would have little effect. With the more slowly absorbed *p*-tolyl PAS, the 1-hour level is markedly affected by excretion rate, and when this is reduced by pre-feeding, the 1-hour level is enhanced.

The blood level increases obtained in human volunteers are also best accounted for on the basis of a decreased rate of excretion, the difference between day 15 and day 1 blood levels being greatest, 3, 4 and 5 hours after a dose, and tending to increase throughout the day (Figs. 1, 2 and 3). A further experiment designed to compare PAS blood levels after prolonged administration of calcium 4-benzamidosalicylate and sodium PAS has been recently reported[12] in which similar blood level increases were demonstrated. These results, although based on small numbers are nevertheless significant and indicate that blood level determinations with drugs intended for long-term administration should be made after a period of continuous treatment, and not after a single dose or a series of doses spaced over one day.

Although the mechanisms by which lowered excretion rate of PAS occurs has not been explained, the similarity in chemical structure of the PAS metabolite 4-aminosalicyluric acid (I) and the known renal-blocking



I



II

drug, 4-aminohippuric acid (II) suggests that 4-aminosalicyluric acid may also be acting as a renal-blocking agent and thereby leading to an increased blood concentration of its precursor, PAS.

From the data reported in this paper, *p*-tolyl PAS is shown to be of low toxicity, only minor toxic symptoms appearing after a dose of 1560 mg./kg. daily for 3 months in rats. This, together with the fact that it is tasteless and produces sustained PAS blood levels approaching those achieved with equivalent doses of sodium PAS, indicates that this substance fulfils many of the criteria for a clinically acceptable PAS derivative.

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REFERENCES

1. Gibson and Nagley, *Tubercle*, 1955, **36**, 209.
2. Jeker, Lauener, Regli and Friedrich, *Amer. Rev. Tuberc.*, 1959, **79**, 351.
3. Bavin, Drain, Seiler and Seymour, *J. Pharm. Pharmacol.*, 1952, **4**, 844.
4. Litchfield, *J. Pharmacol.*, 1949, **96**, 399.
5. Newhouse and Klyne, *Biochem. J.*, 1949, **44**, VII.
6. Frederiksen, Jensen, Mørch and Tybring, *Acta. pharm. tox., Kbh.*, 1957, **14**, 58.
7. Way, Smith, Howie, Weiss and Swanson, *J. Pharmacol.*, 1948, **93**, 368.
8. Banerjea, Drain, Overton and Seymour, *J. chem. Soc.*, 1952, 3861.
9. British Patent 713,178.
10. Torning, Jensen and Klaer, *Acta. Tuberc. scand.*, 1958, **35**, 87.
11. Doub, Schaefer, Bambas and Walker, *J. Amer. chem. Soc.*, 1951, **73**, 903.
12. Drain, Lazare and Tattersall, *Tubercle*, 1959, **40**, 201.

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

The discussion revealed the lack of specificity of the methods of estimation, and the doubtful validity of blood PAS levels. The compounds were soluble to the extent of 10 mg./100 ml. and would probably hydrolyse very slowly at pH 7. It was suggested that steric factors and the configuration of the molecules might account for the differences in activity of the aryl and alkyl esters.

THE ASSAY OF ANTI-HAEMOPHILIC GLOBULIN

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The Biggs, Eveling and Richards assay of anti-haemophilic globulin is examined and some modifications discussed. The statistical design and analysis is offered in a simple form suitable for routine application. The relationships of some defects in the assay to the property of the phospholipid are discussed. It is shown that the method is satisfactory if certain precautions are taken, and if the interpretation is carried out objectively by mathematical methods. Serious errors can arise from neglect of these precautions. Modified assays are described, one of which could probably be used in cases of serious difficulty with reagents. The other apparently depended on the properties of a particular batch of prothrombin. The method was abandoned and is reported here because it is the only modification that gave promise of being more accurate than the original. It is emphasised that the assay is based on hypotheses that have been satisfactorily verified, but that are well recognised to be incomplete. The course of the reaction is by no means clear, and it is unlikely that the assay can accurately be used to compare materials widely dissimilar in qualitative properties.

In the original assay of anti-haemophilic globulin (A.H.G.) published by Biggs, Eveling and Richards[1] a system of reagents is used consisting of isolated but impure blood coagulation factors. From these factors thromboplastin is generated in a quantitative manner related to the quantity of A.H.G. in the system. It can therefore be used for the assay of A.H.G. by measuring the amount of thromboplastin as indicated by the clotting time of a recalcified plasma substrate.

In the method first published the assay was interpreted by comparing the clotting time of the unknown material with a standard curve based on a long series of measurements of another batch of material used as a standard. When we began to use this method in 1954, we found that the relationships between the clotting time and concentrations of A.H.G. were expressible by the commonly observed linear correlation of the logarithm of the dose and the logarithm of the effect, and this has now become standard practice[2].

It is the purpose of this paper to describe the detailed modifications in technique and analysis that we have made to the original method. Some of the difficulties which are now arising in its application are not due to defects in the method, which is a good example of a bioassay, but to the fact that the use of statistical methods of analysis is not common enough in haematology. One result of this is that unsuitable experimental designs are sometimes used, and the magnitude of the errors may be underestimated or overestimated but is rarely measured. We can also contribute some observations on the necessary properties of the reagents used in the assay, although much remains obscure in this complicated field.

The Assay

It is unnecessary to describe the assay process in detail, since the existing accounts are adequate; but to make the present paper comprehensible the following is a brief description.

A system is set up in which Factor V, serum containing Factor VII and Christmas Factor, a phospholipid prepared from human brain, A.H.G. and calcium chloride are allowed to react for 10 minutes. The thromboplastin formed is related to the concentration of A.H.G. and is assayed by adding portions of the incubation mixture to a substrate, consisting of human citrated or oxalated plasma, simultaneously re-calcified, and observing the clotting time. A standard preparation is treated in the same way at approximately the same time, and the results are interpreted by comparing the clotting times in the manner described below.

The Technique of the Test

All reagents including A.H.G. dilutions are stored in the ice bath throughout the assay, since in the course of our investigations we sometimes found that significant changes took place when reagents were kept at room temperature for some hours.

The standard and unknown A.H.G.'s to be assayed are wetted at the same time and when dissolved made up to 1 in 10 with saline. One in 100 dilutions are made from all these and kept on ice.

For a 6-point analysis, doubling dilutions from 1 in 400 to 1 in 3,200 of standard are prepared (the three best consecutive points being chosen for the final mathematical analysis). For a 4-point analysis quadrupling dilutions are in general the best, and in this case 1 in 400 and 1 in 1,600 are appropriate. These dilutions are prepared with a 1 ml. bulb pipette, adding saline from a 10 ml. graduated pipette.

All tubes are mixed by inversion 6 times. When diluting the next sample to be assayed, a new standard must be diluted at the same time.

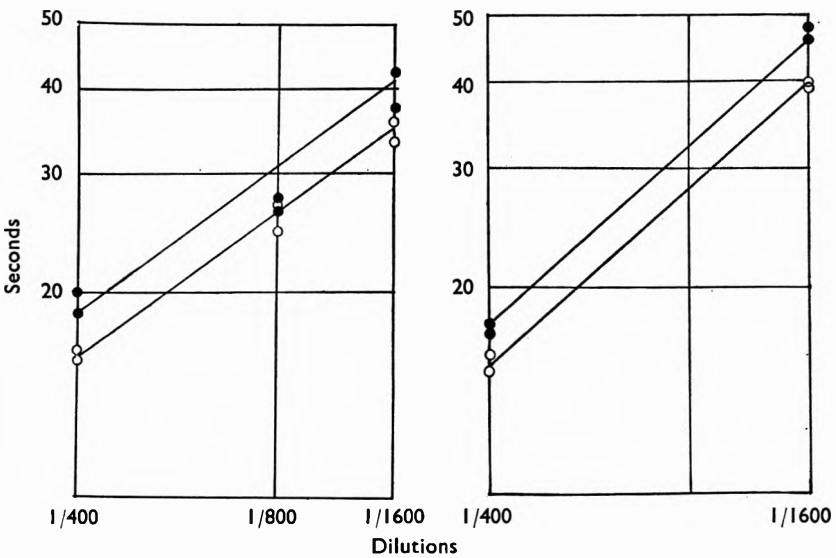
Using Pasteur pipettes graduated at 0·1 ml., incubation mixtures consisting of 0·1 ml. phospholipid, 0·1 ml. Factor V and 0·1 ml. serum, are made up and 0·1 ml. of each A.H.G. dilution (discarding the first 0·1 ml. taken up in the pipette and starting at the weakest dilution) is added to the 8 tubes in order. It is observed that if more than 8 tubes are used in one test the last ones may be erratic, and in the 6-point assay, where we actually test duplicates of 4 dilutions and discard one, we divide the assay into two halves, each with one set of the duplicates. Statistical analysis by the methods of Bliss and Marks[3] showed that the error due to lapse of time between the two halves of the assay was not significant unless the time was long. The order of the tubes is then randomised in the rack by one of the well-known methods. A convenient practical scheme is to write the numbers "S1", "S2" and so on, on the ends of corks, keep these in the pocket and take out one at a time to decide the order.

0·1 ml. $CaCl_2$ solution is added at 1 minute intervals to the tubes in their random order. As soon as the calcium chloride is added to a tube it is shaken and replaced in the water bath at 37°.

A clotting tube containing 0·1 ml. of plasma is put in the water bath at about the ninth minute. 0·1 ml. of the incubation mixture from the first tube is pipetted and discarded. Then 0·1 ml. is taken and added to the plasma tube simultaneously with 0·1 ml. $CaCl_2$ solution starting the stopwatch at the same time. The tube is taken out of the water bath, dried quickly and held in a suitable position, revolving it backwards and forward until a web is perceived. The watch is then stopped and the time entered serially in the note-book, previously prepared to agree with the particular random order in use. The process is repeated on the remaining tubes at one minute intervals.

Statistical Analysis

A simple method of plotting on double logarithmic paper is illustrated in Figures 1 and 2. For rapid use in a clinical laboratory, this may be all that is necessary and, in fact, a *good* assay analysed graphically, rarely



FIGS. 1 and 2. Illustrations of 6 point and 4 point Assay. The points are plotted directly from the experimental results and a pair of parallel lines drawn in by eye to lie evenly among the points. The ratio of the potencies of the two samples, assumed to be present in equal concentrations by weight, is given by the ratio of the readings on the dilution scale corresponding to any point on the time scale.

provides a result significantly different from that given by mathematical methods. One of the inherent difficulties of this assay, particularly when it is being used to compare materials both containing A.H.G. but which are not otherwise identical (for example, normal human plasma and animal A.H.G.) is that the lines drawn may not be parallel. As is well known, this indicates a qualitative difference between the materials and it must be stressed that the advice of the statisticians cannot safely be ignored—they have provided tests of validity which eliminate assays in

Scheme for duplicated 4-point Analysis (Gaddum. *Quadrupling dilutions.* Batch No. *206C.* Date: *7.6.56.*

	U1	U2	S1	S2		
	2041	*5997*	*2504*	*6628*		
	1818	*5925*	*2355*	*6776*		
Sum	*3859*	*11922*	*4859*	*13404*	Sum of	
Diff.	*223*	*72*	*149*	*148*	Diffs.	
Mean	*1929*	*5966*	*2429*	*6702*	*592*	= Q
$\frac{Q}{4{\cdot}52}$	$= \frac{592}{4{\cdot}52}$				$= 130$	= S
$\frac{S^2}{2}$	$= \frac{16900}{2}$				$= 8450$	= V or A
	$\frac{(U1 + U2) - (S1 + S2)}{2}$	$= \frac{7895 - 9131}{2}$			$= -618$	= F
	$\frac{(U2 + S2) - (U1 + S1)}{2}$	$= \frac{12668 - 4358}{2}$			$= 4155$	= E
	$(U2 + S1) - (U1 + S2)$	$= 8395 - 8631$			$= -236$	= G
	$= \frac{E}{-0{\cdot}6020}$	$= \frac{4155}{-0{\cdot}602}$	$= -6900$			$= b$
$\frac{F}{b}$	$= \frac{-618}{-6900}$	$= 0{\cdot}0895$				= M
Antilog M = potency ratio = *1·228*						= R
Validity Tests						
Parallelism	$t = \frac{G}{2\sqrt{V}}$	$= \frac{-236}{184}$			$= 1{\cdot}28$	$t = 2{\cdot}78$
$V(b) = \frac{V}{0{\cdot}36}$	$= \frac{8450}{0{\cdot}36}$	$= 23400$				$= V(b)$
$g = \frac{V(b) \times 7{\cdot}72}{b^2}$	$= \frac{23400 \times 7{\cdot}72}{47500000}$				$< 0{\cdot}1$	g

Fiducial Limits. Use appropriate alternative according to value of g.

$g > 0{\cdot}1$

$$2 + \frac{gM}{1-g} \pm \frac{2{\cdot}78}{b(1-g)} \sqrt{A(1-g) + V(b)\,M^2}$$

$g < 0{\cdot}1$

$$2 \pm \frac{2{\cdot}78}{b} \sqrt{A + V(b)\,M^2}$$

Log Limits = *2 ± 0·035 = 1·965 − 2·035*
Limits = *92 − 108* per cent

FIGS. 3A and 3B. The simplification results from a process of standardisation of procedure, which allows variables to be treated as constants. The omission of the characteristic of the logarithms is possible because in practice all the times in seconds have two digits.

The entry "$t = 2{\cdot}45$" is only a reminder that the calculated value in the validity tests must not exceed this number.

Values of g greater than 0·1 rarely occur. When they do, the assay is generally useless and has to be repeated. The left-hand formula is, therefore, hardly ever used but can sometimes give limited information in the case of urgency. Its value is to reveal the extent of the unreliability of unsatisfactory assays. Having arrived at the potency ratio R the activity of the unknown is simply found by multiplying the activity of the standard by this number, making due allowance for dilutions.

ASSAY OF ANTI-HAEMOPHILIC GLOBULIN

Scheme for duplicated 6-point Analysis (Gaddum). *Doubling dilutions.* Batch No. 59.
Convert all times to logs, and omit characteristic. Date 9.2.58

	U1	U2	U3	S1	S2	S3		
	3010	*4346*	*6274*	*2148*	*4314*	*5490*		
	2695	*4216*	*5752*	*1987*	*3945*	*5263*		
Sum	*5705*	*8562*	*12026*	*4135*	*8259*	*10753*	Sum of	
Diff.	*315*	*130*	*522*	*161*	*369*	*227*	Diffs.	
Mean	*2852*	*4281*	*6013*	*2067*	*4129*	*5376*	*1724*	= Q

$$\frac{Q}{6{\cdot}78} = \frac{1724}{6{\cdot}78} = 254 = S$$

$$\frac{S_2}{2} = \frac{64500}{2} = 32250 = V$$

$$\frac{(U1 + U2 + U3) - (S1 + S2 + S3)}{3} = \frac{13146 - 11572}{3} = 524 = F$$

$$\frac{(U3 + S3) - (U1 + S1)}{4} = \frac{11389 - 4919}{4} = 1617 = E$$

$$\frac{(U3 + S1) - (U1 + S3)}{2} = \frac{8080 - 8228}{2} = -74 = G$$

$$U3 + U1 - 2\,(U2) = 8865 - 8562 = 303 = H_1$$

$$S3 + S1 - 2\,(S2) = 7443 - 8258 = -815 = H_2$$

$$-\frac{E}{0{\cdot}3010} = -\frac{1617}{0{\cdot}3010} = -5370 = b$$

$$\frac{F}{b} = -\frac{524}{5370} = -0{\cdot}0975 = M$$

Antilog M = potency ratio $= 0{\cdot}7989 = R$

Validity tests $t = 2{\cdot}45$

Parallelism $\dfrac{G}{\sqrt{V}} = \dfrac{74}{180} = 0{\cdot}412$

Curvatures $\dfrac{H_1}{\sqrt{6v}} = \dfrac{303}{441} = 0{\cdot}688$

$\dfrac{H_2}{\sqrt{6v}} = \dfrac{816}{441} = 1{\cdot}85$

$= A$

$A = \dfrac{2V}{3} = 21500$ $\quad V(b) = \dfrac{V}{0{\cdot}364} = 89000 = V(b)$

$\dfrac{V(b)\,t^2}{b^2} = \dfrac{89 \times 6}{29000} = \dfrac{535}{29000} = <0{\cdot}1 = g$

Fiducial limits. Use appropriate alternative according to value of g.

$g > 0{\cdot}1$

$$2 = \frac{gM}{1-g} \pm \frac{2{\cdot}45}{b\,(1-g)}\sqrt{A\,(1-g) + V\,(b)\,M^2}$$

$g < 0{\cdot}1$

$$2 \pm \frac{2{\cdot}45}{b}\sqrt{A + V\,(b)\,M^2}$$

Log Limits $= 2 \pm 0{\cdot}0680 = 1{\cdot}9320 - 2{\cdot}0680$
Limits $= 86 - 117$ per cent

which the fundamental requirement of similarity of response is objectively tested.

Since a proposed standard for A.H.G. is the activity in 1 ml. of normal pooled human plasma, difficulties over validity and relevance may arise. In our opinion there is need for a standard consisting of a certain quantity of dry A.H.G. of agreed activity. It is true that as with other biological standards, there is no guarantee that this will remain stable for prolonged periods. The ultimate standard may therefore well be the average level of A.H.G. in the population at large, which is unlikely to change, but a comparison at rare intervals to establish that the dry standard has not significantly changed is a very different thing from the regular use of a standard material which is qualitatively different from the drug itself. Material called A.H.G. is without doubt extremely complex and plasma far more so. Unless the material under test is as like as possible to the standard, trouble is almost sure to arise.

Another "disease" of this assay is a poor slope. Since slope and random error enter into the calculation of limits as their squares, the combination of poor slope and not very good agreement between duplicates can make an assay almost useless. This is not easy to detect without mathematical analysis. The B.P. provides suitable methods of analysis based upon the paper by Gaddum[4]. Both Gaddum and the B.P. have rightly provided for a variety of schemes to cover many requirements, but in doing so have left the potential user with a choice I feel he would often prefer not to make. In Figures 3*a* and 3*b* are given therefore the only two schemes we have found it necessary to use, so simplified that technicians work out their own results as a matter of routine. If these forms were accepted as the right way of working out an A.H.G. assay without worrying about the reasons, a considerable advance would be made towards objectivity.

Reagents

All the organic reagents used in this assay, including the A.H.G., are complex and imperfectly purified native clotting factors. The system is not far removed from life, and it is a tribute to the perspicacity of its authors that they have reproduced in a test tube much of what happens in the body. It is, therefore, no surprise to find that the properties of the reagents vary. The serum (providing Christmas factor and factor VII) and factor V rarely give difficulties once the proper concentration and incubation time for any particular batch have been found. The trouble is almost always due to the phospholipid, and it is possible that preparations are in use that would not give reasonably accurate results if mathematical analysis were used to measure the errors.

The original work at Oxford and by ourselves was carried out on one particular batch of phospholipid. When this preparation was repeated, both at Oxford and in these laboratories, it was found that the necessary properties were not always present. The slope was often too poor to be useful because the times at high concentrations of A.H.G. were too long, and those at low concentrations too short. Acting on the hypothesis that there were two components in the phospholipid, one an accelerator and

the other an inhibitor, we prepared numerous varieties of phospholipid from the brains of cattle, pigs, horses, man and also from soya beans, and fractionated some of them by various methods. In one such attempt we prepared an inhibitor from human brain by the method of Rapaport, Aas, and Owren[5]. By using a soya bean phospholipid[6] in the incubation mixture and this inhibitor in the substrate, we produced a system which although not as good as the brain phospholipid, gave reasonably accurate results in trial. We then fractionated crude brain phospholipids and confirmed the observations of Newlands[7] to the extent that some of these fractions are inhibitory and some are accelerators, as we had imagined. The position is made more complicated by the fact that a phospholipid may be an accelerator at one concentration but an inhibitor at another. We had limited success by combining these purified fractions in the assay. We have also found that two crude preparations, one of which gives a short time but a poor slope, and the other gives long times, could give good results by being mixed in suitable proportions (Fig. 4). The mixture is less stable than a suitable crude preparation. We have observed that a deterioration in slope of a mixture can be corrected by adding more of the material giving long times.

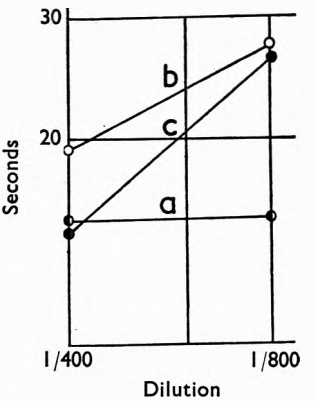


FIG. 4. The difference in properties of phospholipids in this system:
(*a*) is a preparation of high activity in the generation of thromboplastin, but quite useless in the assay for it is not sensitive to varying concentrations of A.H.G,
(*b*) of improved sensitivity, but with rather poor activity,
(*c*) is a mixture of the two and shows high sensitivity and good times.

When these observations are compared with the repeatedly observed fact that deterioration of a phospholipid suspension almost always shows itself in a deterioration of slope, it seems possible that we are indeed witnessing the destruction, probably by oxidation, of an inhibitor less stable than an accelerator but both essential for the assay.

There is, however, an alternative hypothesis. We found during the investigations on soya bean phospholipids that a fraction having no thromboplastic activity, when tested by the one stage prothrombin test, developed activity when incubated in the usual mixture with serum, factor V and calcium chloride, but without A.H.G. The activity was of the same order as that of A.H.G. and a logarithmic relation between concentration of phospholipid and clotting time was found. It is, therefore possible that certain specimens of phospholipids may be active in this system, even in the absence of A.H.G. and could account for unsatisfactory slopes for, in effect, a constant quantity of activity would be added at all dilutions thus reducing the supposed ratios between the dilutions.

We believe that we now have a limited degree of control over the properties of the phospholipid component of the incubation mixture, but

much further work is required before it will be possible to say that all the difficulties have been removed, if, indeed, this ever happens. At this point we must emphasise that preparations that are highly active as shown by very short clotting times may be quite useless in the assay, which requires, not short times, but a high sensitivity to the variations in A.H.G. concentrations. Figures 1, 2 and 4 show a selection of some of our experimental results illustrating the foregoing descriptions.

The Substrate

After trying numerous combinations of the reagents prepared from various animals with various substrates, we concluded that the only suitable substrate was fresh human plasma. Bovine plasma has been tried but in our hands this has always given poor slopes, and the experience at Oxford was similar. We once succeeded in devising a system somewhat resembling that of Wolf[8] in which bovine prothrombin was added to the incubation mixture so that thrombin was produced there. Excellent results were obtained, more accurate than the original method, because of the greatly improved slope and capable of being tested on a bovine substrate. When this batch of prothrombin was used up, we attempted to reproduce it with quite unsatisfactory results and we had to abandon this line of enquiry.

Acknowledgements. I am grateful to Drs. R. G. Macfarlane, R. Biggs, E. Bidwell and R. F. Welch for many helpful discussions and gifts of material. Most of the early experimental work was carried out by Mrs. Freda Gerson and many later experiments by Misses Doreen Cockerell, and Ann Booth and Mr. Rolf Lundgren.

REFERENCES

1. Biggs, Eveling and Richards, *Brit. J. Haemat.*, 1955, **1**, 20.
2. Biggs and Macfarlane, *Human Blood Coagulation and its Disorders*, 2nd Edn., Blackwell, Oxford, 1957.
3. Bliss and Marks, *Quart. J. Pharm. Pharmacol.*, 1939, **12**, 82; 182.
4. Gaddum, *J. Pharm. Pharmacol.*, 1953, **5**, 345.
5. Rapaport, Aas and Owren, *J. Lab. clin. Med.*, 1954, **44**, 364.
6. Newlands and Wild, *Nature, Lond.*, 1955, **176**, 885.
7. Newlands, Ph.D. Thesis, University of Cambridge, 1957.
8. Wolf, *Brit. J. Haemat.*, 1956, **2**, 367.

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

The state of purity of the phospholipid might account for the difficulties encountered with this substance. A limit to the lysophosphatide fraction might be desirable. Hens' eggs were suggested as the most reliable source of phosphatide.

THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURY COMPOUNDS

PART III. THE EFFECTS OF CERTAIN SULPHYDRYL COMPOUNDS ON *E. coli* I

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The antibacterial activity of cysteine, glutathione, thioglycollate, dimercaprol and horse serum to *E. coli* I has been studied. Incorporation of these materials into solid media has little adverse effect upon the number of cells multiplying on it, but in the diluents used for preparing dilutions for counting the effect is more marked. Dimercaprol produces the largest reduction in the number of cells subsequently multiplying. Dimercaprol and thioglycollate are believed to exert their effect by retarding the metabolic processes. Concentrations of the materials suitable for quantitative work involving viable counts have been determined.

SEVERAL workers[1–6] have demonstrated an inhibitory action of cysteine on *E. coli*, especially in a chemically defined medium; the concentration of cysteine responsible being variously reported between 0·002 and 0·2 per cent. The inhibitory action has been ascribed to a prolongation of the lag phase[4,8] when *E. coli* is grown aerobically in chemically defined medium, but does not occur when grown "semi-aerobically"[4] or in broth cultures[8]. Italian workers[6] showed cysteine to cause a temporary bacteriostasis of *E. coli*, the amount and duration of suppression depending on the concentration of cysteine.

Indian workers[2] reported that 1 in 5×10^4 of glutathione had some inhibitory effect upon the growth of *E. coli* whereas Pratt[7] reported that it exhibited a stimulating effect on the growth of this organism, in a chemically defined medium. In a later communication[8], however, it was stated to have little effect on the growth of the organism.

Dubnoff[5] stated that concentrations of about 0·1 mg./ml. of thioglycollic acid were toxic to "wild" types of *E. coli* but Sykes and others[9], in experiments with a variety of organisms, demonstrated that growth retardation was apparent only in concentrations of thioglycollate above 0·5 per cent.

Renoux and Roux[10] reported the bacteriostatic concentration of dimercaprol to *E. coli* was greater than 25 μg./ml., and Berry and Jensen[11] showed that a concentration of 1 in 27·5 showed no bacteriostatic effect on this organism.

An account of the various specific and non-specific factors present in serum which can react with bacteria *in vitro* is given by Wilson and Miles[12].

In view of the various and often controversial findings on the effects of the sulphydryl compounds on *E. coli* it was deemed necessary to determine

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whether these compounds exhibited any antibacterial activity towards the strain used in this work. It may be seen that any effect of the compounds on bacteria may be due to the compound *per se* or to a change in the oxidation-reduction potential produced by the compound.

Experimental and Results

Five substances were selected as possible antagonists of the antibacterial action of mercuric chloride[13]; cysteine hydrochloride, glutathione and thioglycollic acid (monothiols), dimercaprol (a dithiol) and normal horse serum. The first four substances are more or less pure compounds containing a known proportion of sulphydryl, whilst horse serum is a natural product having a small and presumably variable sulphydryl content.

Solutions of cysteine hydrochloride, glutathione and thioglycollic acid were adjusted to pH 7·0 with sodium hydroxide solution; dimercaprol solutions were not adjusted. Solutions were sterilised by filtration with the exception of thioglycollate which was autoclaved[14]. Dilutions of horse serum were aseptically prepared. Solutions of the sulphydryl compounds were prepared on the day of use and assayed by titration with potassium iodate[15].

The bacteriostatic activity of the antagonists towards *E. coli* I was determined by both the liquid and solid dilution methods.

After incubation, crystals of (presumably) cystine were visible at the base of all tubes of liquid medium containing 0·2 per cent or more of cysteine; in the solid medium containing the same concentrations, the cystine was in the form of fine particles dispersed throughout the medium.

Plates containing 0·05 per cent or more of dimercaprol became heavily clouded with oxidised dimercaprol during the overdrying process; this made the surface of the medium rather water-repellant, the drops of culture medium added as inocula being poorly absorbed and tending to coalesce. With 0·25 per cent or more of dimercaprol the solid medium was so opaque as to make it difficult to see some surface colonies, and growth in the liquid medium could not be determined even with the aid of transmitted light. Berry and Jensen[11] overcame this difficulty by determining the pH of the medium; they showed that growth in peptone water containing dimercaprol was accompanied by a pH shift towards alkalinity, but in the experiments recorded here, recourse was made to a biochemical test (indole production with Ehrlich's rosindole reagent). Control experiments showed that the dimercaprol did not interfere with the sensitivity of the test.

The results of these experiments showed that the antagonists were not bacteriostatic to *E. coli* I in the following concentrations: cysteine, dimercaprol and glutathione 0·5 per cent, thioglycollate 1 per cent and horse serum 50 per cent. (It is possible, however, that with the lower concentrations of cysteine and dimercaprol, because of their ease of oxidation, it was the bacteriostatic activity of the oxidised form which was being determined.)

Effect of the Antagonists upon the Viable Count of a Suspension of E. coli *I*

The possible effects of the proposed antagonists were investigated on this organism by means of two sets of experiments. The first consisted of a comparison of the viable counts obtained on peptone agar medium containing various concentrations of the antagonists; the second was similar but the antagonists were incorporated into the dilution blanks and the counts on plain peptone agar were compared. Samples of the final mixture for plating were allowed to stand at room temperature for

TABLE I

EFFECT OF THE ANTAGONISTS ON THE VIABLE COUNT OF *E. coli* I. MEAN NUMBER OF COLONIES DEVELOPING PER DROP AREA IN A SURFACE-VIABLE COUNT

	Concentration of antagonist, per cent					
Horse serum	20	15	10	5	2·5	0
Antagonist in plate	24·24	24·40	23·00	24·06	24·14	23·54
,, ,, diluent	25·76	25·97	25·74	26·19	27·21	20·43
As above, after standing ½ hr	27·46	26·55	26·81	26·12	27·04	13·70
Cysteine	0·25	0·20	0·15	0·10	0·05	0
In plate	25·98	26·04	26·48	26·06	26·14	25·48
In diluent	20·75	19·20	21·22	20·03	19·87	17·48
,, ,, after ½ hr	19·40	19·10	19·50	20·10	19·70	15·95
Glutathione	0·25	0·20	0·15	0·10	0·05	0
In plate	26·40	25·58	26·32	25·44	25·32	25·38
In diluent	25·50	24·82	25·65	25·65	24·63	24·60
,, ,, after ½ hr	24·90	24·50	25·85	24·50	24·10	21·60
Thioglycollate	0·25	0·20	0·15	0·10	0·05	0
In plate	25·12	26·76	27·20	25·54	25·98	25·10
In diluent	20·70	24·48	25·58	26·33	26·48	25·87
,, ,, after ½ hr	15·90	19·80	21·10	24·10	26·80	24·40
Dimercaprol	0·20	0·15	0·10	0·05	0·01	0
In plate	23·66	23·34	22·16	23·68	24·24	22·72
In diluent	8·62	9·83	12·03	18·77	20·60	20·28
,, ,, after ½ hr	1·40	4·60	8·88	20·70	20·90	18·71

half an hour and the resultant counts compared with those plated immediately after preparation. Each set of experiments was performed over five days, one antagonist being investigated each day. A suspension of *E. coli* I was prepared and adjusted to contain approximately 2×10^9 viable organisms per ml. This suspension was stored at 4° when not in use. The counting method was similar to that described by Miles and Misra[16]. The results for the two series of experiments are shown in Table I, where the mean number of colonies developing per drop area has been calculated from at least 10 replicate counts.

The colonies developing on medium containing 0·25 or 0·20 per cent and even 0·1 per cent of thioglycollate were much smaller than those developing on plain peptone agar plates; this has been interpreted as due to a possible retarding of the metabolic processes of the bacterial cells caused by the high concentration of thioglycollate. A similar reduction in colony size was observed with medium containing 0·15 per cent or more of dimercaprol. Difficulty was encountered with overdried plates containing 0·1 per cent or more of dimercaprol as the insoluble oxidation

product made absorption of the drops unsatisfactory. These concentrations of dimercaprol resulted, after incubation, in a medium too opaque to allow counting of the colonies by the usual illuminated counting box and recourse was made to illumination by obliquely reflected light. This was not particularly satisfactory and it was felt that there was a possibility of much larger counting errors.

In the experiments where the antagonists were incorporated into the diluents, the drop volumes of the serum dilutions differed markedly from those of water, due primarily to the difference in density, and the counts were adjusted to take this into account. Concentrations of 0·20 and 0·15 per cent of dimercaprol appeared to exert a strong retardation of the growth processes of the cells as, in many cases, colonies took 18–24 hours to become visible and were much smaller than those developed on the control plates.

Discussion

As already noted, the experiments were not all carried out at the same time and hence comparison of the results between the antagonists is not valid, neither is that between the two series. The results within a series for a particular antagonist may however be compared.

Incorporation of the antagonists into the medium had little effect upon the number of organisms subsequently growing upon it. A comparison of the highest and lowest mean counts by Student's *t* test for any dilution of a particular antagonist revealed no significant differences ($P = 0{\cdot}95$).

With the antagonists incorporated into the dilution blanks the results were as follows. In all dilutions of serum the mean count was significantly greater than that obtained using water as the diluent, but there was no significant difference between the counts with the different dilutions. A comparison of the mean counts of samples plated immediately with those allowed to stand for half an hour before plating revealed reasonable maintenance of viability but showed a significant increase with the 20 per cent serum dilution and a marked decrease in the case of the aqueous control. This material has the disadvantages of frothing and allowance must be made for its drop volume being different from that of water.

Cysteine and gluthathione in concentrations up to 0·25 per cent appear to have little effect upon the viability of *E. coli* I. With cysteine a comparison of means in both the dilutions plated immediately and those allowed to stand showed that those containing cysteine were not significantly different from each other. No significant difference was apparent between the mean colony counts from any concentration of glutathione tested whether plated immediately or allowed to stand.

The use of a 0·25 per cent thioglycollate solution as the diluent resulted in a significant reduction in viability, even when the dilution was plated immediately after preparation. In dilutions allowed to stand before plating a significant reduction in viability occurred with all concentrations above 0·1 per cent. Prolonged contact with 0·1 per cent or more of thioglycollate causes a reduction in the number of viable cells, and those still viable multiply more slowly than usual.

Dimercaprol in concentrations above 0·1 per cent causes a marked reduction in the viable count of the organism, and this reduction was even more pronounced in the case of those dilutions not plated immediately. High concentrations probably exert their effect by retarding the metabolic processes.

In all cases the aqueous controls showed reduced viability if not plated immediately.

From these experiments the following concentrations of the proposed antagonists appear to be suitable for quantitative work involving viable counts of *E. coli* I:

Normal horse serum	up to 20	per cent			
Cysteine	„ „ 0·25	„ „	(about 15 mM)		
Glutathione	„ „ 0·25	„ „	(„ 8 mM)		
Thioglycollate	„ „ 0·1	„ „	(„ 10 mM)		
Dimercaprol	„ „ 0·05	„ „	(„ 5 mM)		

References

1. Sahyun, Beard, Schultz, Snow and Cross, *J. infect. Dis.*, 1936, **58**, 28.
2. Das Gupta and Guha, *Ann. Biochem.*, 1941, **1**, 14.
3. Simon, *Brit. J. exp. Path.*, 1948, **29**, 202.
4. McLaughlan, *Canad. J. Microbiol.*, 1957, **3**, 967.
5. Dubnoff, *Arch. Biochem. Biophys.*, 1952, **37**, 37.
6. Benigno, Visentini and Franceschini, *Ric. sci.*, 1955, **25**, 828.
7. Pratt, *J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 621.
8. Pratt and Okano, *ibid.*, 1957, **46**, 433.
9. Sykes, Royce and Hugo, *J. Pharm. Pharmacol.*, 1952, 4, 366.
10. Renoux and Roux, *Ann. Inst. Pasteur*, 1951, **80**, 633.
11. Berry and Jensen, *Acta pharm. int.*, 1951, **2**, 221.
12. Wilson and Miles, "*Topley and Wilson's Principles of Bacteriology and Immunology*", 4th Edn., Edward Arnold, London, 1955.
13. Cook and Steel, *J. Pharm. Pharmacol.*, 1959, **11**, 666, 729.
14. Cook and Steel, *ibid.*, 1959, **11**, 216.
15. Steel, *ibid.*, 1958, **10**, 574.
16. Miles and Misra, *J. Hyg., Camb.*, 1938, **38**, 732.

THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURY COMPOUNDS

PART IV. QUALITATIVE ASPECTS OF THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURIC CHLORIDE

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The stoichiometric relations between mercuric chloride and the sulphydryl antagonists have been examined. In liquid cultures, cysteine, glutathione, dimercaprol and thioglycollate are effective as inactivators of mercuric chloride in quantities close to the theoretical amounts. When used to revive mercuric chloride-treated cells, larger amounts are needed, and horse serum is ineffective. The results obtained with *E. coli* I suggest it is not so resistant to the action of mercuric chloride over long periods as are Gram-positive organisms. Cells treated with mercuric chloride in the presence of a nutrient medium derive some protection from the constituents of the medium. Qualitative experiments show dimercaprol to be the most efficient antagonist and thioglycollate the least.

THE first recorded use of an antagonist was by Geppert in 1889 who used ammonium sulphide to overcome the activity of mercuric chloride. Despite this early work, the literature abounds with reports of the antibacterial activity of mercury compounds in which no antagonist was used, and it is advisable to consider such work with caution. Most workers have used either sulphides or sulphydryl compounds, but sometimes other substances have been used. The introduction of a medium containing thioglycollate for the cultivation of anaerobes[1] also did much to encourage the use of antagonists for the inactivation of mercurial compounds.

EXPERIMENTAL AND RESULTS

Volumetric estimations were made to determine the combining ratios of mercuric chloride with the antagonists, used in Part III[2]. The antagonist solutions were freshly prepared and adjusted to pH 7, with the exception of dimercaprol solution, and each solution was standardised by titration with potassium iodate solution[3]. The antagonist solution was titrated with mercuric chloride solution, using a freshly prepared ammoniacal solution of sodium nitroprusside as indicator. From these results the ratio of the number of molecules of antagonist reacting with one molecule of mercuric chloride was calculated as follows. Cysteine hydrochloride 2·004, glutathione 2·043, thioglycollic acid 2·023, and dimercaprol 1·007. Thus, within the limits of experimental error, it may be stated that one molecule of mercuric chloride reacts with two sulphydryl groups.

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In the first series of experiments to demonstrate antagonism, the procedure was to inoculate peptone water mixtures containing a known bacteriostatic concentration of mercuric chloride and varying concentrations of the antagonists with *E. coli* I and to determine, after incubation, whether bacteriostasis had been achieved. Simultaneously, an accurate determination of the bacteriostatic value of mercuric chloride was made.

In mixtures containing dimercaprol or serum, both of which produced an opacity or precipitate, the indole reaction was used to determine the presence or absence of growth.

Full tables of replicated results for the five antagonists are available but have not been reproduced here. A typical set of results for one

TABLE I

THE EFFECT OF GLUTATHIONE ON THE BACTERIOSTATIC VALUE OF MERCURIC CHLORIDE AGAINST *E. coli* I

Mercuric chloride (μM)	Glutathione (μM)							
	0	10	20	50	100	200	300	400
60	−	−	+	+	+	+	+	+
90	−	−	−	−	+	+	+	+
120	−	−	−	−	−	+	+	+
150	−	−	−	−	−	+	+	+
200	−	−	−	−	−	−	+	+
250	−	−	−	−	−	−	−	+

+ = growth, − = no growth

antagonist and only one replicate is shown in Table I; the bacteriostatic concentration of mercuric chloride against *E. coli* I under the same experimental conditions was 55 μM.

From these results, the following deductions were made. Bacteriostasis is expected if the concentration of mercuric chloride is 55 μM or more, and where growth occurs, the effective concentration of mercuric

TABLE II

NUMBER OF MOLECULES OF THE ANTAGONIST WHICH ANTAGONISE ONE MOLECULE OF MERCURIC CHLORIDE

Antagonist	Not antagonistic	Minimum antagonistic
Cysteine	1·7	2·1
Dimercaprol	0·7	1·1
Glutathione	1·9	2·1
Thioglycollic acid	1·9	2·2

chloride is presumed to have been reduced below 55 μM. From a knowledge of the concentration of mercuric chloride and of antagonist in each tube, some approximate value for the combining ratio of the two substances can be calculated. Thus, with 90 μM mercuric chloride, growth occurred in the presence of 100 μM glutathione and so the glutathione has reduced the effective concentration of mercuric chloride by, at least, 90 − 55 = 35 μM, hence one molecule of mercuric chloride has been antagonised by 100 ÷ 35 = 2·86 molecules of glutathione. Similarly, with 90 μM mercuric chloride, no growth occurred in the presence of 50 μM glutathione and so the effective concentration of mercuric

chloride had not been reduced by 35 μM and one molecule of mercuric chloride was not antagonised by 1·43 molecules of glutathione. The results of such calculations for all replicates and concentrations of the antagonists are given in Table II. With horse serum, the concentrations necessary to reduce the effective concentrations of mercuric chloride below their bacteriostatic value were not as constant in replicate experiments as with the sulphydryl compounds. Serum, 20 to 25 per cent, reduced the mercuric chloride concentration by about 100 μM, 10 to 20 per cent by about 60 to 70 μM and 5 to 10 per cent by about 30 to 40 μM.

Dimercaprol showed little antagonistic activity when mixed with the peptone water before addition of the mercuric chloride solution, but if added after the mercuric chloride was present there was good antagonism. This is possibly related to some reaction of the dimercaprol with the medium, the reaction of dimercaprol with proteins having been reported[4]. With glutathione, the results read after 24 hours incubation were usually unchanged by further incubation. With the other antagonists, however, growth often became apparent only after 72 hours. This is believed to be due to the possible role of glutathione in the metabolism or nutrition of the cells rather than to a quicker antagonistic action.

The experimental results obtained were in reasonable agreement with those obtained by titration of mercuric chloride with the antagonists.

Effect of Adding the Antagonist After the Bacteria have been in Contact with Mercuric Chloride

In the previous experiments the antagonist and mercuric chloride were both present in the system before the addition of the bacteria and hence the antagonist was acting in a true antagonistic manner, that is as an inactivator. It is of more value to know whether the antagonist can act as a reviver of organisms which have been in contact with mercuric chloride and, if so, after what time.

In this second series of experiments, the bacteria were added to peptone water containing varying concentrations of mercuric chloride. After 30 minutes contact at 20°, the antagonist was added, the tubes incubated at 37° for 7 days, and then examined for growth. Calculations as before gave the following mean minimum number of molecules of antagonist required to antagonise one molecule of mercuric chloride. Cysteine 3·2, glutathione 2·3, thioglycollate 3·4 and dimercaprol 1·4. With horse serum, only one revival (or recovery) occurred in the series. In view of its virtual inefficiency as a reviver, even after only 30 minutes contact with mercuric chloride, its use was abandoned.

Recovery of Organisms after Longer Exposure to Mercuric Chloride

In the experiments reported the organisms had been in contact with the antibacterial agent for up to 30 minutes before the addition of the antagonist. Such conditions are unlikely to be obtained in practice, for example in testing samples for sterility. The time during which organisms held in a state of bacteriostasis might be expected to survive was now investigated.

A series of tubes containing 10 ml. of peptone water with varying concentrations of mercuric chloride was inoculated with one drop of a suspension of *E. coli* I (equivalent to about 2×10^7 organisms) and incubated at 37° for 24, 48 or 72 hours. After incubation, tubes containing growth were discarded. From each negative tube, half of the contents were transferred to a sterile test-tube as a control. To the remainder, known amounts of antagonist solution were added, and both tubes were re-incubated. The presence or absence of growth was recorded after a further 7 days incubation.

With concentrations of 50 to 100 μM mercuric chloride and 0·2 to 20 mM antagonist solution, only one revival was obtained. This was with 80 μM mercuric chloride and 0·2 mM cysteine added after 24 hours.

Further similar experiments were made but with the addition of an extra 5 ml. of peptone water after the antagonist had been added. In these experiments only 3 recoveries were noted, one each with cysteine, dimercaprol and thioglycollate. No concentration of glutathione produced any recoveries.

Recovery of Mercuric Chloride-treated Organisms in Aqueous Media

Experiments were now made by adding the test organisms to mercuric chloride solution and the antagonist and culture medium after varying periods of contact. The following methods of adding the antagonist solution and the medium were considered possible. First, by allowing a short period for reaction of the antagonist and the mercuric chloride and then adding the medium. Second, by adding the antagonist solution immediately followed by the medium, and third by adding the medium into which the antagonist had been incorporated. Fildes[5] showed the reaction between mercuric chloride and sulphydryl compounds was practically immediate and so the first method was not used. The third method is an easier procedure, involving less manipulations, but the oxidation of sulphydryl compounds has been shown to occur more rapidly in dilute solution, and the second method was finally adopted.

One drop of a suspension of *E. coli* I was added to each of a series of tubes containing (A) 1 ml. of 500 μM and (B) 4 ml. of 125 μM mercuric chloride solution. The tubes were kept at 20°. At 5-minute intervals up to 1 hour, 1 ml. of antagonist solution was added to replicate reaction mixtures followed by 5 ml. of double strength peptone water and, with (A) only, 3 ml. of sterile water. The tubes were then incubated at 37° for 7 days and examined for growth. The concentrations of antagonists were 10, 5, 2·5 and 1 mM for the monothiols and 5, 2·5, 1·25 and 0·5 mM for dimercaprol. These concentrations corresponded, in the final mixtures, to molecular ratios of sulphydryl to mercuric chloride (—SH : Hg ratio) of 20, 10, 5, and 2 : 1 respectively.

The results showed that recovery always occurred where the —SH : Hg ratio was 10 or 20 : 1 and in most instances where it was 5 : 1. Only dimercaprol and thioglycollate caused any recovery at a 2 : 1 ratio. Bearing in mind the variation in resistance of the organism to bactericides, a similar variation in resistance to bacteriostatic action may account for

the few recoveries obtained with thioglycollate at a 2:1 ratio. With dimercaprol at the same ratio, most of the recoveries occurred in the first 20 minutes of testing and these are significant.

As it was possible to obtain recovery after a one hour contact with mercuric chloride by the use of a sufficiently large excess of antagonist, experiments were made to ascertain the time after which recovery was not possible. The experimental procedure used 5 ml. of 50 or 100 μM

TABLE III

RECOVERY OF MERCURIC CHLORIDE-TREATED *E. coli* I FROM AQUEOUS SUSPENSION, USING THIOGLYCOLLATE AS ANTAGONIST

Percentages of Replicates showing Recovery

Contact time, hours	Mercuric chloride concentration			
	50 μM		100 μM	
	Reaction temp.			
	20°	37°	20°	37°
1	100	100	100	50
1·25	100	100	80	40
1·5	100	80	60	30
1·75	100	30	70	10
2	100	40	90	30
2·5	100	20	70	20
3	100	10	50	15
3·5	90	0	30	20
4	70	0	20	0
over 4	0	0	0	0

mercuric chloride solution, inoculating with one drop of a suspension of *E. coli* I and keeping the reaction mixtures at 20 or 37°. After varying contact times, 5 ml. of double strength peptone water, containing a concentration of thioglycollate equivalent to twenty times the mercuric chloride concentration, was added and the mixtures incubated at 37° for 7 days. (The peptone water-thioglycollate mixtures were freshly prepared.) Table III shows the results, expressed as the percentage of replicates showing recovery.

DISCUSSION

The dual action of inactivation and revival has been discussed, in relation to thioglycollate, by Berry[6] who pointed out that not all inactivators would function as revivers. Comparison of the results obtained when the antagonist was present in the reaction mixture (Table II) with those when it was added later shows that a significantly higher amount of antagonist is necessary for revival than for inactivation.

The poor revival obtained with horse serum suggests its lack of specificity. It is believed that when mercuric chloride is mixed with serum before addition of the bacteria, combination of the mercurial compound with serum proteins occurs which reduces the antibacterial activity. When the bacteria have been in contact with mercuric chloride it is possible that adsorption of the mercury on the cells, or its combination with them, is by bonds of such a strength that the serum proteins are incapable of causing its removal. On the other hand, if reversal is brought

about by sulphydryl groups then serum is of little value as a reviver, as its sulphydryl content is very low[7]. It is possible that serum may be of more value when a more specific sulphydryl-reacting mercuric compound is investigated; Davison[8] found normal horse serum to be a suitable antagonist for phenylmercuric nitrate.

The results obtained for the recovery of organisms after longer exposure to mercuric chloride are not in agreement with reports in the literature on the time organisms can be held in a state of bacteriostasis and still show viability when a suitable antagonist is added. Fildes[5] however reported that *E. coli* could not be revived after 17 hours contact with 2×10^{-6} M mercuric chloride at 38°, but his inoculum was only about 1000 organisms.

The following possible explanations are advanced to account for these results. (i) The organisms of the inoculum were dead by the time the antagonist was added, (ii) insufficient antagonist was added, although in some cases the $-SH : Hg$ ratio was 400 : 1, (iii) nutrients were not available for re-growth of the treated cells; this implies that the combination of mercuric chloride with constituents of the medium was such that the antagonist was incapable of reversal. In experiments of this nature it is not feasible to greatly increase the concentration of antagonist as it might itself have an adverse effect upon the organisms[2].

From the results where additional medium was added after the antagonist, the hypothesis that the medium was "poisoned" by the excess mercuric chloride has not been proved valid. It appears that *E. coli* I is not a suitable organism for experiments of this kind as revival was rarely possible after only 24 hours contact with mercuric chloride. Examination of other organisms should give information whether this phenomenon is peculiar to *E. coli* I or is applicable to other Gram-negative organisms. Most literature reports on the revival of mercurial-treated organisms are confined to Gram-positive ones.

Recovery and growth of injured cells may occur if it is possible for the cells to rid themselves of mercury which has entered into combination with their substance. Wyss[9] believed that cells could recover spontaneously from bacteriostasis produced by limiting concentrations of heavy metals, by the production of more sulphydryl groups which displaced the metal from its attachment on the sulphydryl group in the active site of enzymes or gene proteins. If the organisms are treated with mercuric chloride in the absence of a culture medium, no re-growth will be possible and it is expected that cells held in a state of bacteriostasis will not be revivable, by addition of an antagonist, for as long a period as cells in a nutrient medium.

The experiments show that it is possible to antagonise the effect of mercuric chloride on *E. coli* I after a limited contact time and to cause revival and recovery, by addition of a sulphydryl compound. For recovery, an excess of the sulphydryl compound is required. Under the experimental conditions used, cysteine, glutathione and dimercaprol were effective in ratios of $-SH : Hg$ of 5 or more to 1; thioglycollate was effective at a 10 : 1 ratio. Of these compounds, dimercaprol was the most efficient antagonist and thioglycollate the least. It appears that even the

lowest theoretical concentration of dimercaprol prevented mercuric chloride from exerting its action upon bacteria over a certain time.

Opinions vary on the most suitable antagonist for mercurial compounds, although all agree on the necessity of having an excess of antagonist present. Fildes[5] found the antagonistic action of glutathione was greater than that of thioglycollate, on a molecular basis, whilst Bailey and Cavallito[10] found cysteine to produce more rapid reversal than glycylcysteine, *N*-acetylcysteine or sodium thioglycollate. Brewer[11] showed dimercaprol, mercaptoethanol and sodium formaldehyde sulphoxalate to be no more effective than sodium thioglycollate and Powell[12] reported the reviving power of sodium thioglycollate to be greater than that of dimercaprol, but believed this might be due to decomposition of the latter. Woodbine[13], however, considered dimercaprol and glutathione were more effective than either cysteine or thioglycollate.

The necessity of an excess amount of antagonist over the theoretical quantity may be due to any or all of the following factors. (i) a loss of antagonist by atmospheric oxidation, (ii) the necessity of lowering the oxidation-reduction potential of the system to a level suitable for the growth of the revived cells, (iii) after removal of the mercuric chloride, essential sulphydryl groups contained within the bacterial cells may be in the oxidised state and additional antagonist may be required for their reduction, (iv) the complexes formed between mercuric chloride and the sulphydryl compounds are capable of ionisation[14] and excess antagonist may be necessary to overcome the effects of this ionisation. Sulphydryl antagonism of the antibacterial action of mercuric chloride does not involve competitive inhibition, which is regarded as occurring when two substances, which cannot chemically interact, compete for a common site on an enzyme. It is well known that the antagonists used can combine chemically with mercuric chloride.

References

1. Brewer, *J. Amer. med. Ass.*, 1940, **115**, 598.
2. Cook and Steel, *J. Pharm. Pharmacol.*, 1959, **11**, Suppl. *157 T.*
3. Steel, *ibid.*, 1958, **10**, 574.
4. Spray, *Biochem. J.*, 1947, **41**, 360.
5. Fildes, *Brit. J. exp. Path.*, 1940, **21**, 67.
6. Berry, *J. Pharm. Pharmacol.*, 1951, **3**, 689.
7. Steel, *Brit. vet. J.*, 1959, **115**, 281.
8. Davison, *J. Pharm. Pharmacol.*, 1951, **3**, 734.
9. Wyss, *Bacterial Physiology*, edit. by Werkman & Wilson, Academic Press Inc., New York, 1951.
10. Bailey and Cavallito, *J. Bact.*, 1948, **55**, 175.
11. Brewer, *Ann. N. Y. Acad. Sci.*, 1950, **53**, 211.
12. Powell, *J. gen. Microbiol.*, 1950, **4**, 330.
13. Woodbine, *J. Pharm. Pharmacol.*, 1950, **2**, 376.
14. Steel, *Ph.D. Thesis*, University of London, 1959.

After Dr. Steel presented both papers there was a DISCUSSION.

A STUDY OF FACTORS AFFECTING THE INACTIVATION OF QUATERNARY AMMONIUM COMPOUNDS ON AGAR

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Pharmaceutical preparations for topical use containing antibacterial quaternary ammonium compounds have been reported to show reduced *in vitro* activity when tested by the seeded agar plate method. The "resistance" met by an alkyldimethylbenzylammonium chloride, reported by Heineman[1], was ascribed by Sherwood[2] to the presence of agar. Tobie and Ayres[3], and later Hoogerheide[4], attributed the reduced activity to a slow rate of diffusion owing to the high molecular weights of quaternary ammonium compounds. Phillips[5] amplified this, suggesting that "the ionic aggregates of quaternary ions may be physically too large to pass through the agar (gel) network." Quisno, Gibby and Foter[6] showed that granular agar and agar in sol were equally effective in adsorbing quaternary ammonium compounds and claimed that discrepancies between tests with solid and liquid media could not therefore be explained by differences in diffusion rates. Other factors affecting the development of inhibition zones by quaternary ammonium compounds on seeded agar plates have now been investigated.

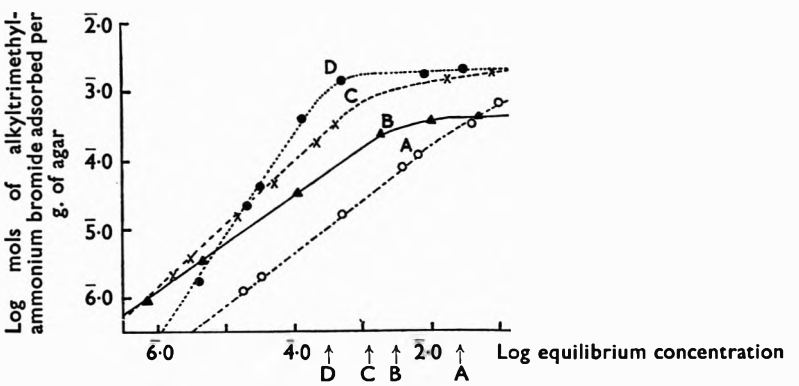

Fig. 1. The adsorption of alkyltrimethylammonium bromide homologues by New Zealand agar at 37°.

A Dodecyl-trimethylammonium bromide
B Tetradecyl- „ „ „
C Hexadecyl- „ „ „
D Octadecyl- „ „ „

The respective critical micelle concentrations[12] are indicated by the arrows.

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The adsorption of an homologous series of alkyltrimethylammonium bromides by granular agar was examined by a method adapted from that of Quisno and others[6]. Preliminary work showed that the particle size of the granules made no significant difference to the amount of quaternary ammonium compound adsorbed or to the time required to reach equilibrium. *Davis* New Zealand agar (44/60 mesh) was used throughout: 250 mg. of the agar was stirred for two hours at 37° with 10 ml. of quaternary ammonium compound solution, filtered and the filtrate assayed by the method of Few and Ottewill[7]. This was repeated for concentrations of each homologue from 10^{-1} to 10^{-5} molar and results are given in Figure 1 in the form of Freundlich adsorption isotherms.

TABLE I

THE ADSORPTION OF A SERIES OF ALKYLTRIMETHYLAMMONIUM BROMIDE HOMOLOGUES ON NEW ZEALAND AGAR

(Calculated from Fig. 1)

Homologue	Molecular weight	Freundlich adsorption isotherm for concentrations below the critical micelle concentration	Critical micelle concentration (from Harkins[12])	Approximate maximum weight adsorbed moles/g. of agar
Dodecyl-	308	$X/M = 0{\cdot}006C^{0{\cdot}75}$	0·015	0·001
Tetradecyl-	336	$X/M = 0{\cdot}018C^{0{\cdot}72}$	0·0034	0·00045
Hexadecyl-	364	$X/M = 0{\cdot}448C^{0{\cdot}98}$	0·001	0·0018
Octadecyl-	393	$X/M = 560{\cdot}0C^{1{\cdot}6}$	0·00025	0·002

For each of the four homologues there is a point on the isotherm above which the adsorption process ceases. This point appears to correspond to the critical micelle concentration for any one of the quaternary ammonium homologues and a similar type of phenomenon was observed by Weatherburn and Bayley[8] who investigated the adsorption of surface-active agents on cotton and wool. These authors attributed the saturation achieved to the adsorption of unassociated ions, the ionic aggregates, or micelles, taking little part in the process at or above the critical micelle concentration. The amount of any one homologue adsorbed per unit mass of agar increases with increase of molecular weight up to the critical micelle concentration (Table I) and the maximum amount adsorbed per gram of agar is about 10^{-3} mols.

Whilst this figure may not apply to quaternary ammonium compounds differing in structure from the alkyltrimethylammonium bromide series, its order of magnitude appeared sufficiently small to suggest that, if the interpretation of Quisno and others[6] is tenable and unequal diffusion rates are not responsible for the reduced activity, zones of inhibition should be obtainable on seeded agar plates. In a further search for suitable conditions a number of different quaternary ammonium compounds were investigated using a method based on the seeded agar well-plate method. The materials employed were chemically pure and a series of aqueous solutions from 10^{-2} to 10^{-5} molar were prepared. These were placed in wells prepared in nutrient broth solidified with 1 per cent New Zealand agar seeded with *Staphylococcus aureus*. After incubation the

zones of inhibition were measured and the results plotted as a function of the concentration.

Results obtained to date, in addition to those reported in Figure 2, indicate that if the dose-response curves are interpreted as suggested by Humphrey and Lightbown[9] the "critical" concentration of any quaternary ammonium compound on an agar plate is about the same as the bacteriostatic concentration in liquid media.

The addition of 10^{-4} molar Sky Blue FF to 1 per cent agar gel enables the detection of micelle aggregates within the gel, such a dye being a micelle indicator (Corrin and Harkins[10]), and this is evidence which seems

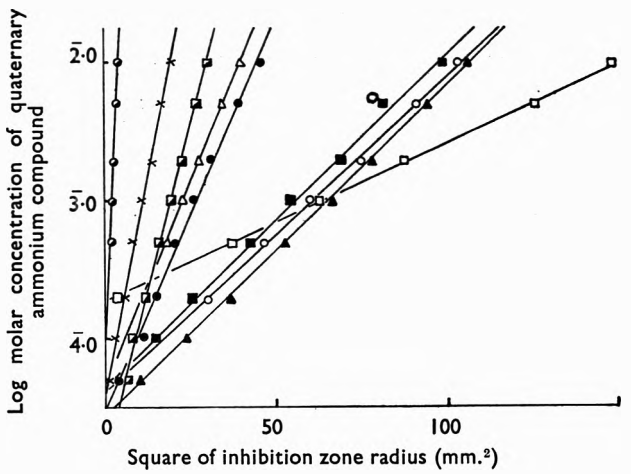

FIG. 2. Dose-response curves for a number of quaternary ammonium compounds when tested against *Staph. aureus* by means of the agar well-plate method.

○ = Dodecyldimethylbenzylammonium chloride. ● = Tetradecyldimethylbenzylammonium chloride. ◔ = Hexadecyldimethylbenzylammonium chloride. □ = Dodecyltrimethylammonium bromide. ■ = Tetradecyltrimethylammonium bromide. ◢ = Hexadecyltrimethylammonium bromide. × = Hexadecylpyridinium chloride. △ = Domiphen bromide. ▲ = Phenoctide chloride ("Octaphen").

to disprove the hypothesis of Phillips[5]. It is not known if the micelles diffuse from the solution in contact with the gel or form at any point within the gel where the concentration of diffusing ions has exceeded the critical micelle concentration. The agar diffusion technique for the investigation of antibody-antigen reactions[11] involving high molecular weight entities indicates that large molecules can diffuse through agar gels. The molecular weights of most antibacterial quaternary ammonium compounds lie between 300 and 400 and these are below the molecular weights of common antibiotics, for example, streptomycin base (582), chlortetracycline hydrochloride (516), oxytetracycline hydrochloride (497) and tetracycline hydrochloride (481).

It is concluded that the inactivation of antibacterial quaternary ammonium compounds by agar is not sufficient to preclude the investigation of preparations containing these compounds by the conventional

seeded agar plate methods. By refrigerating the plates after adding the material under test, but before incubation, the size of the zones of inhibition can be increased and the use of such a technique should enable the testing of antibacterial quaternary ammonium compounds to be undertaken in a manner similar to that employed for materials containing antibiotics.

REFERENCES

1. Heineman, *J. Amer. pharm. Ass.*, 1937, **26**, 711.
2. Sherwood, *J. Bact.*, 1942, **43**, 778.
3. Tobie and Ayres, *ibid.*, 1944, **47**, 109.
4. Hoogerheide, *ibid.*, 1945, **49**, 277.
5. Phillips, *Nature, Lond.*, 1947, **160**, 55.
6. Quisno, Gibby and Foter, *J. Amer. pharm. Ass., Sci. Ed.*, 1946, **35**, 317.
7. Few and Ottewill, *J. Colloid Science*, 1956, **11**, 34.
8. Weatherburn and Bayley, *Textile Research J.*, 1952, **22**, 797.
9. Humphrey and Lightbown, *J. gen. Microbiol.*, 1952, **7**, 129.
10. Corrin and Harkins, *J. Amer. chem. Soc.*, 1947, **69**, 679.
11. Ouchtrelony, *Acta path. microbiol. scand.*, 1953, **32**, 231.
12. Harkins, *Surface Films*, Rheinhold, New York, 1952.

After Mr. Groves presented the communication there was a DISCUSSION.

Short Communication

THE COMPARATIVE PROTECTIVE EFFECTS OF DEGRADED CARRAGEENIN AND ALUMINIUM HYDROXIDE ON EXPERIMENTALLY PRODUCED PEPTIC ULCERATION

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Acute peptic ulceration of the duodenum can be produced experimentally in guinea pigs within 24 hours of the administration of a large dose of histamine in beeswax-oil provided a covering dose of an antihistamine is given to protect the animals from acute shock.

Degraded carrageenin, a sulphated polysaccharide (Ebimar) has been shown to react with mucus lining the gastric mucosa and in low concentrations to interfere with peptic digestion. It provides complete protection against duodenal ulceration produced by the above method[1,2]. In the present experiments we have compared its effects on ulcerogenesis with those of aluminium hydroxide.

Methods. Male guinea pigs (300–400 g.) were prepared for experiment by depriving them of food from 5 p.m. on the day before the experiment. The next day at 4.30 p.m. the animals were injected intraperitoneally with promethazine 7·5 mg., followed within half an hour by an intra-muscular injection of histamine acid phosphate 30 mg./kg. in a 10 per cent (w/v) beeswax : arachis oil vehicle. The suspension was made by triturating 1·5 g. histamine acid phosphate (200 mesh) with the vehicle and adjusting the volume to 50 ml.

The animals were studied in groups of five, and the drugs were administered in 2 ml. doses intra-oesophageally at 3 hour intervals from 6 hours before to 21 hours after the histamine injection. Ebimar was given in aqueous solutions containing 20, 10, 5, 1, or 0·2 per cent w/v. Aluminium hydroxide was administered as liquid gel containing the equivalent of 4, 2, or 1 per cent Al_2O_3 w/w. The control group were given 2 ml. of water at the same intervals.

The animals were killed 24 hours after the histamine injection. The stomach and duodenum were removed, fixed in formol saline, and the degree of damage was assessed numerically according to the scheme, Table I.

Results. The degree of damage in the stomach and duodenum in each group of five animals is recorded in Table I. The maximum score if all five animals were severely damaged would be 15 for the stomach, 15 for the duodenum, giving a total score of 30.

It is seen in the control group which received water only that the maximum score was reached in the duodenum. Considerable protection from ulceration in the duodenum was apparent in the groups treated with

10 per cent and 20 per cent sulphated polysaccharide, and in those treated with 2 per cent and 4 per cent aluminium hydroxide.

Less protection was provided with the lower concentrations of each of these drugs. In contrast, however, the sulphated polysaccharide afforded greater protection against stomach ulcers than aluminium hydroxide.

It was observed that considerable caking of aluminium hydroxide had occurred in the stomachs of the groups receiving 4 per cent and 2 per cent gel, but this was not evident in the group receiving 1 per cent gel.

TABLE I

NUMERICAL ASSESSMENT OF THE DEGREE OF GASTRIC AND DUODENAL DAMAGE IN SULPHATED POLYSACCHARIDE AND ALUMINIUM HYDROXIDE-TREATED ANIMALS. CONTROL GROUP RECEIVED ONLY WATER

	Sulphated polysaccharide per cent					Aluminium hydroxide gel per cent as Al_2O_3			Water
	20	10	5	1	0·2	4	2	1	
Stomach ..	3–4	2–3	2–3	3	0–1	4–6	8–9	10	9–10
Duodenum	0	3	8	14–15P	12–13P	0	0	6–7	14–15P
Totals ..	3–4	5–6	10–11	17–18P	13–14P	4–6	8–9	16–17	23–25P

Key: 0 No damage,
1 very slight damage, for example, one or two small lesions revealed on careful examination,
2 immediately obvious but not extensive damage,
3 immediately obvious and widespread damage; perforation is indicated by P.

In the sulphated polysacharide treated groups, particularly those receiving the higher concentrations, a fine gelatinous film was observed over the mucosa, especially in the lower third of the stomach and in the duodenum.

Discussion. The method of producing acute peptic ulceration in the above experiments allows the slow release of histamine over a prolonged period. In the guinea pig, histamine stimulates the secretion of a large volume of highly acid gastric juice[3], and it is believed that this factor is primarily responsible for the mucosal damage[4,5]. The results in the aluminium hydroxide series may be said to be in fair agreement with this theory.

From the results of experiments as yet unpublished, we have evidence that in the histamine stimulated guinea pig 2 ml. of 4 per cent aluminium hydroxide gel greatly reduces the acidity of the gastric juice for 2 to 3 hours; 2 per cent gel causes some reduction, but with 1 per cent gel the free acid is only very slightly reduced. It may, therefore, be assumed that over the 27 hour period during which aluminium hydroxide was administered in the present experiments little, if any, free acid is likely to have been present in the stomach or duodenum in the group receiving the 4 per cent gel. This assumption was confirmed by the fact that at post-mortem examination no free acid was detected in the gastric juice in the group receiving 4 per cent gel, but free acid was present in the stomachs of the groups receiving the 2 per cent and 1 per cent gels. As already indicated (Table I), duodenal lesions occurred only in the group given 1 per cent gel. It is noteworthy that despite the accumulation of substantial

amounts of aluminium hydroxide in the stomachs of the group receiving 2 per cent gel, a distinct reaction for free acid (Topfer's reagent) was obtained in the post-mortem juices.

On the basis of the aluminium hydroxide experiments, where increasing damage appears concurrently with increase in free acid, it is not possible to assess the relative importance of the acid and pepsin in the pathogenesis of the ulcerative lesions described. Although these experiments might suggest that the protective action of aluminium hydroxide gel was because of its antacid properties, it must also be appreciated that the anti-peptic activity of this compound may play an important role. Nevertheless, the use of antacids in peptic ulcer therapy is well established and the demonstration of a protective action by the antacid in these experiments perhaps not too surprising. It is interesting to observe on the other hand, that degraded carrageenin which possesses no antacid activity should afford protection to the extent reported.

The evidence we have already obtained about the properties of degraded carrageenin suggests that it has at least two main actions[1,2], and we consider that these actions may be intimately concerned in the protective effects of the sulphated polysaccharide used in these experiments. Degraded carrageenin reacts with mucoprotein in a manner to be expected of polyanionic substances in acid environment. We tentatively conclude that the complex formed between mucoprotein in the mucus lining the stomach and degraded carrageenin serves to enhance the physical protection afforded by mucin. In addition, the degraded carrageenin confers anti-peptic properties on this complex.

References

1. Anderson and Watt, *J. Physiol.*, 1959, In the press.
2. Anderson and Watt, *J. Pharm. Pharmacol.*, 1959, **11**, 318.
3. Watt, *Quart. J. exp. Physiol.*, 1955, **40**, 364.
4. Code, *Surg. Clin. N. America*, 1943, **23**, 1091.
5. Watt, M.D. Thesis, University of Aberdeen, 1956.

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

THE EFFECT OF PROTOVERATRINE A ON POTASSIUM AND CALCIUM ION MOVEMENTS IN MUSCLE AND NERVE

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The effects of protoveratrine A on the efflux of potassium ions (K^+) from frog and rat skeletal muscle and the isolated electrically driven rat heart have been studied. No effect was seen upon the rate of efflux from skeletal muscle but that from the heart was increased. Protoveratrine A increases K^+ uptake from frog skeletal muscle and causes this tissue to release more calcium (Ca^{++}). There was no increase in Ca^{++} release from lobster nerve treated with protoveratrine A. The theoretical implications of these findings are discussed.

The mechanism of action of the veratrum alkaloids is still not fully understood. The ester alkaloids are responsible for the antihypertensive actions and probably act on sensory nerve endings in the lungs, heart and great vessels. Direct actions on the brain and nodose ganglion have also been demonstrated[1]. The veratrum alkaloids sensitise sensory nerve endings and may do so by upsetting the normal ionic balance. There is sensitisation to K^+ in nerve and muscle and when sensitised tissues are stimulated, spontaneous repetitive responses occur[2]. Rosenblueth[3] has suggested that veratrine acts upon nerves by preventing the reabsorption of K^+ released during the spike. Shanes[4] has shown that K^+ is released from nerve exposed to veratrine and Gordon and Welsh[5] have suggested that veratrine may displace Ca^{++} from the cell membrane and so alter its permeability to ions. Harris (personal communication) and Khan and Acheson[6] have not been able to show changes in K^+ flux in muscle or nerve after treatment with veratrum alkaloids. Preliminary investigations[7] measuring absolute concentrations of K^+ have indicated that cardiac muscle, unlike skeletal muscle, shows an increase in K^+ efflux after protoveratrine. This investigation was made to determine whether protoveratrine A altered uptake and efflux of Ca^{++} and K^+ from muscle and nerve and to see whether these effects were related.

Materials and Methods

The composition in mM of the bath fluids used was as follows.

Fenn Ringer's fluid[8]. NaCl, 111·20; KCl, 2·50; $CaCl_2$, 1·80; Na_2HPO_4, 2·50; NaH_2PO_4, 0·50. Locke's solution. NaCl, 153·90; KCl, 5·63; $CaCl_2$, 2·16; $NaHCO_3$, 5·95; dextrose, 5·55. Krebs-Henseleit solution. NaCl, 118·30; KCl, 4·69; $CaCl_2$, 2·61; K_2HPO_4, 1·17; $NaHCO_3$, 14·16; $MgSO_4$, 2·41; dextrose, 11·10. Lobster saline (Robertson, personal communication). NaCl, 490·30; KCl, 8·75; $CaCl_2$, 57·6; $MgCl_2$, 27·4; $NaHCO_3$, 2·48; Na_2SO_4, 31·70.

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Radioactive bath fluids were made by replacing some of the KCl or $CaCl_2$ by a calculated amount of ^{42}KCl or $^{45}CaCl_2$. A 1 mg./ml. stock solution of protoveratrine A was made by dissolving the free base in dilute hydrochloric acid and neutralising the excess acid until the pH was 6·8. The solution was stored at 4° in the dark and diluted as required with the appropriate saline solution.

$^{42}K^+$ Loading of Muscles

A sterile 1·15 per cent w/v solution of ^{42}KCl with a specific activity of 0·15 mc./ml. was obtained from A.E.R.E. Harwell. 1 ml. of this was injected into the dorsal lymph sac of male or female frogs weighing from 25 to 50 g. Male or female albino rats weighing between 150 and 200 g. were given 2 ml. of the solution by intraperitoneal injection. A 2-hour equilibration period was allowed before the animals were killed and the sartorius or soleus muscles removed.

$^{42}K^+$ Release from Frog Sartorius Muscle

The frog sartorius muscles are thin and flat: they can be easily removed intact and with care paired muscles can be obtained which differ in weight by less than 2 mg. $^{42}K^+$ efflux was measured by suspending the muscles in three successive 10 ml. samples of Fenn Ringer's fluid followed by five successive samples containing 2 or 10 μg./ml. of protoveratrine A, allowing 10 minutes for each immersion. The control muscle was passed through a parallel series without protoveratrine A. Each muscle was then blotted dry and dissolved in 10 ml. of concentrated nitric acid and the total radioactivity of the solution determined. The radioactivity of the bathing solution was estimated by means of an Ekco M6 Geiger-Muller tube using a scaler of conventional pattern. The readings were corrected for background and lost counts and expressed as counts per minute.

$^{42}K^+$ Release from Rat Soleus Muscle

The soleus muscle was chosen because it has a thin flattened structure. Muscles were suspended in successive 10 ml. volumes of Krebs-Henseleit solution at 37° aerated with 95 per cent O_2 and 5 per cent CO_2. The method used was similar to that for frog sartorius muscle.

$^{42}K^+$ Release from Rat Cardiac Muscle

Hearts were rapidly removed from ^{42}K-loaded rats, washed free from blood and perfused with oxygenated Locke's solution at 37° by Langendorff's method. To avoid drug-induced variations in rate the heart was driven electrically at a rate of 120 per minute by means of square pulses of 10 volts amplitude and 10 msec. duration. The radioactive effluent from the heart was allowed to flow under slight negative pressure through an Ekco F 10 liquid flow counting tube connected to a recording ratemeter. Variations in radioactivity were shown as changes in the gradient of the recorded curve. When the rate of exchange between the $^{42}K^+$ of the heart and the K^+ of the perfusion fluid was constant, 2 or 5 μg. of protoveratrine A were injected into the aortic cannula.

$^{42}K^{+}$ Uptake by Frog Sartorius Muscle

Paired sartorius muscles differing in weight by not more than 2 mg. were used. The control was immersed for 10 or 20 minutes in 10 ml. of Fenn Ringer's fluid containing $^{42}K^{+}$ at 19° agitated and aerated by a stream of O_2. The test muscle was immersed in a similar solution containing 10 μg./ml. of protoveratrine A. The muscles were removed from the solutions, drained and each flat surface washed for 5 seconds in a stream of Fenn Ringer's fluid to remove $^{42}K^{+}$ adherent to the surface. The radioactivity of the muscle could now be assumed to be due to intra- or intercellular $^{42}K^{+}$. Radioactivity was measured by exposing each flattened muscle surface for 1 minute, 1·5 cm. away from an Ekco GM4 aluminium

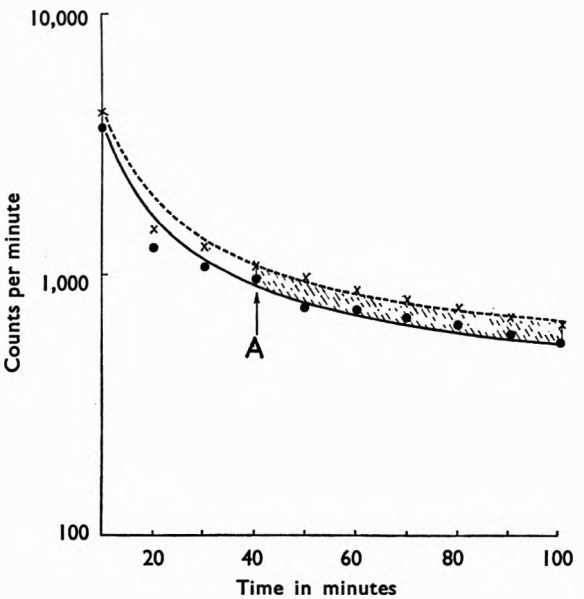

FIG. 1. $^{42}K^{+}$ release from frog sartorius muscle. At A, 10 μg./ml. protoveratrine A added to the test.
×—× Test. ●—● Control.

end-window counter which was connected to a recording ratemeter. The muscles were then returned to the radioactive solution for a further period of 10 minutes and the procedure repeated. Total time of exposure was 3 hours but in some experiments it was increased to 10 hours. At the end of the experiment the total radioactivity of each muscle was determined by dissolving it in concentrated nitric acid and counting in a liquid counter (see above).

$^{45}Ca^{++}$ Release from Frog Sartorius Muscle

Paired sartorius muscles were soaked for 3 hours at room temperature in oxygenated Fenn Ringer's fluid of which the Ca^{++} had been replaced by $^{45}Ca^{++}$. The muscles were washed to remove adherent $^{45}Ca^{++}$ and

the experiment continued as described for $^{42}K^{+}$ release but to ensure that $^{45}Ca^{++}$ release was occurring at the same rate from both muscles each was exposed for six successive 10 minute periods to a series of control solutions. The test muscle was then exposed for three successive 10-minute periods to a series of three tubes each containing 10 μg./ml. of protoveratrine A in Fenn Ringer's fluid and finally to two more tubes containing Fenn Ringer. Radioactivity was determined by taking three 0·5 ml. samples from each tube, adding 0·1 ml. of 1 per cent w/v solution of cetrimide (to ensure even spreading) and evaporating to dryness on

TABLE I

$^{42}K^{+}$ RELEASE FROM FROG SARTORIUS MUSCLE AFTER EXPOSURE TO 10 μg./ml. OF PROTOVERATRINE A

1	2	3	4	5
		Residual $^{42}K^{+}$ in muscle counts per min.		
Experiment No.	Area between curves cm.^{2}	control	test	Difference (4—3)
1	+ 4·4	9,721	11,127	−1,406
2	− 1·4	33,584	30,024	+3,560
3	− 9·5	6,027	7,405	−1,378
4	+13·0	22,992	23,836	− 844
5	− 4·1	33,665	33,977	− 272
6	+ 2·3	11,945	10,731	+1,214
7	+14·1	5,515	2,031	+3,484
8	−10·0	6,416	6,255	+ 161
9	− 9·2	12,885	15,082	−2,197

a flat aluminium planchette. The mean radioactivity of each group of three samples was determined by counting with an Ekco EW3H mica end-window counter using a conventional scaler. After correction the results were expressed as counts per minute and multiplied by twenty to give the total count for the solution.

$^{45}Ca^{++}$ Release from Lobster Nerve

The nerve from the cheliped of the common lobster (*Homarus vulgaris*) was dissected and halved. One half was used as the control, the other as the test. The nerves were soaked for 1 hour in lobster saline containing $^{45}Ca^{++}$. After loading, the surfaces were washed for 10 minutes with lobster saline to remove adherent $^{45}Ca^{++}$. The control nerve was exposed for ten successive periods of 10 minutes in a series of planchettes each containing 0·5 ml. of lobster saline. The procedure was repeated for the test nerve but each of the last five tubes also contained , 110 or 100 μg. per ml. of protoveratrine A.

RESULTS

$^{42}K^{+}$ Release from Frog Sartorius Muscle

The amount of $^{42}K^{+}$ taken up by muscles from different frogs varied widely but the individuals from a pair took up corresponding amounts. Variations in the weights of muscles from different frogs and in the radioactivity of the ^{42}KCl made direct comparisons of results difficult. It has been assumed that $^{42}K^{+}$ efflux is proportional to the total K^{+} flux in the

muscle. Data from each experiment was expressed by plotting $^{42}K^+$ efflux against time. The curves obtained from a typical experiment are shown in Figure 1. Differences in $^{42}K^+$ efflux between test and control were obtained by measuring with a planimeter the area subtended by

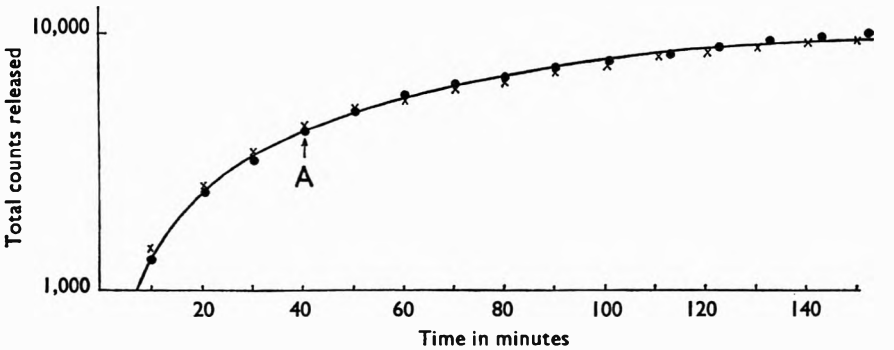

FIG. 2. $^{42}K^+$ release from rat soleus muscle. At A 10 μg./ml. protoveratrine A added to the test. ×—× Test. ●—● Control.

the two curves. When total $^{42}K^+$ efflux from the control was greater than from the test the difference was taken as positive, when less, as negative (see Table I). When analysed using the *t* test the differences in $^{42}K^+$ efflux between control and test were not significant ($P > 0{\cdot}9$). Therefore, 10 μg./ml. protoveratrine A, a concentration reported to be sufficient to influence the physiological and electrochemical behaviour of

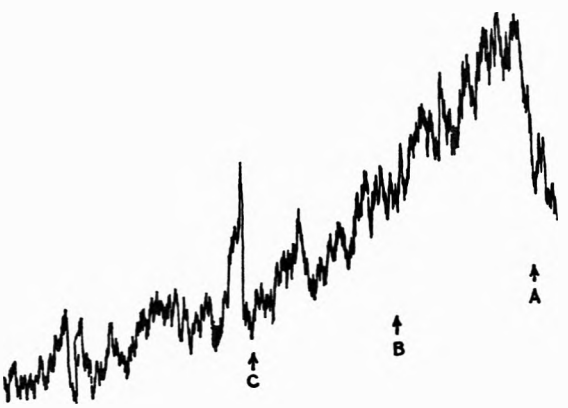

FIG. 3. $^{42}K^+$ release from rat cardiac muscle. Tracing reads from right to left. At A, beginning of experiment. At B, 0·4 ml. control solution. At C, 2 μg. protoveratrine A.

the muscle[1,2] had no effect on $^{42}K^+$ efflux. These results were confirmed using $^{42}K^+$-loaded muscles bathed in Ringer's solution continuously circulated through an FM6 flow counter when bath concentrations of protoveratrine of 1, 10 or 100 μg./ml. failed to increase $^{42}K^+$ efflux.

$^{42}K^+$ Release from Rat Soleus Muscle

The results were treated similarly to those obtained from frog sartorius muscle. 1 or 10 μg./ml. of protoveratrine A had no effect upon $^{42}K^+$ efflux from rat skeletal muscle (Fig. 2). In some experiments muscles were made to contract isometrically by stimulating at a rate of 2 per second with square wave pulses, at 10 volts, 10 msec. duration and a frequency of 100/min. Protoveratrine A had no detectable influence on $^{42}K^+$ efflux in these preparations.

$^{42}K^+$ Release from Rat Cardiac Muscle

Protoveratrine A, 2 or 5 μg., reversibly increased $^{42}K^+$ efflux. As heart rate and cardiac output were constant this effect appeared to be

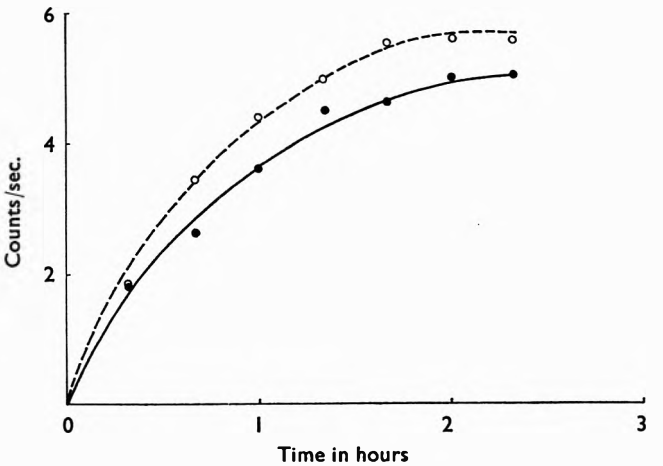

FIG. 4. $^{42}K^+$ uptake by frog sartorius muscle. The test muscle was treated with 10 μg./ml. protoveratrine A.
○—○ Test. ●—● Control.

due to a direct drug action (Fig. 3). These results agree with the findings of Vick and Kahn[10] who used isolated guinea pig hearts treated with veratridine and measured the K^+ concentration of the effluent before and after drug administration.

$^{42}K^+$ Uptake by Frog Sartorius Muscle

The rate and total uptake of $^{42}K^+$ from a labelled medium differed considerably between frogs. The method used here made it possible for the activities of the treated and the control muscles of each pair to be measured and recorded side by side, thus reducing errors of measurement as much as possible. By plotting the total uptake by each muscle against time, curves of an exponential character were obtained (Fig. 4).

In ten experiments the rate and total uptake of $^{42}K^+$ by the protoveratrine treated muscle was always greater than that from the corresponding control muscle. This difference was found to be significant ($P < 0{\cdot}001$).

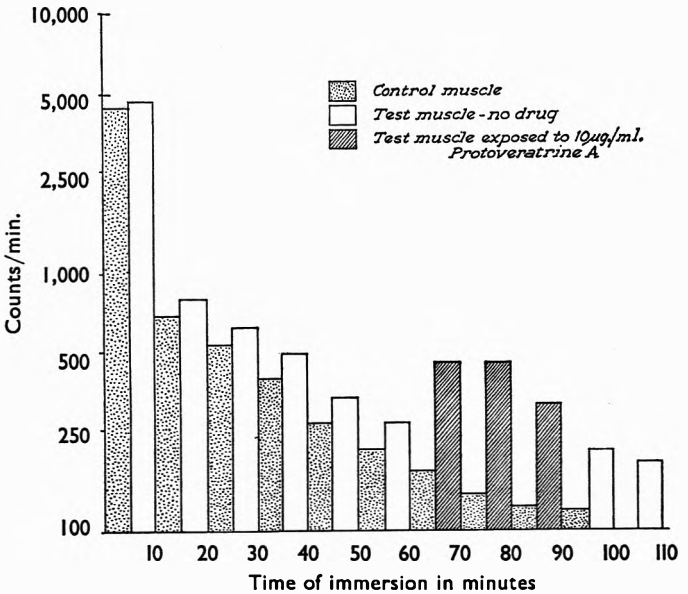

FIG. 5. $^{45}Ca^{++}$ release from frog sartorius muscle.

TABLE II

$^{45}Ca^{++}$ RELEASE FROM FROG SARTORIUS MUSCLE BY PROTOVERATRINE A
10 μg./ml.

1	2		3	4	5	6	7	
	Wt. (mg.) of muscle		Total number of counts released during			Percentage of total released by		Ratio of results in columns 6 to 7
Expt. No.	C = Control T = Test		run	exposure to drug	exposure to control	exposure to drug	exposure to control	
1	C	80	17,085		2,361		13·82	
	T	80	16,180	2,839		17·54		1·27
2	C	36	7,104		1,552		21·84	
	T	37	8,533	2,569		30·01		1·38
3	C	24	9,918		285		2·87	
	T	24	11,579	762		6·58		2·29
4	C	40	2,044		224		1·09	
	T	38	2,030	307		1·51		1·38
5	C	40	2,436		237		0·97	
	T	42	2,335	397		1·70		1·75
6	C	54	5,289		586		1·10	
	T	52	5,698	758		1·33		1·21
7	C	78	7,099		433		6·1	
	T	77	8,543	1,196		14·0		2·29
8	C	148	2,554		153		5·99	
	T	148	2,644	284		10·74		1·79
9	C	129	8,506		801		9·42	
	T	130	11,067	1,114		10·07		1·07
10	C	27	2,849		161		5·65	
	T	28	3,156	293		9·28		1·64

$^{45}Ca^{++}$ *Release from Frog Sartorius Muscle*

The normal rate of $^{45}Ca^{++}$ release from frog sartorius muscle was exponential and similar to that reported by Harris[9]. In ten experiments exposure of the $^{45}Ca^{++}$ loaded muscle to 10 μg./ml. of protoveratrine A led to more $^{45}Ca^{++}$ being released into the bathing fluid than from the control. In seven of these, maximum $^{45}Ca^{++}$ release occurred during the first 10 minute period of exposure and in the remainder during the second 10-minute period. After returning to the normal bathing solution the rate and total $^{45}Ca^{++}$ release fell approximately to control levels (Fig. 5). Paired muscles were comparable with respect to uptake and release of Ca^{++} but muscles from different frogs of the same weight showed no correlation. The total counts per minute obtained from the muscle during exposure to the drug were expressed as the total counts per cent, released during the entire experiment. In each experiment more $^{45}Ca^{++}$ was released from the protoveratrine-treated muscle than from the control (Table II). The results indicate that 10 μg./ml. protoveratrine A significantly increases ($P < 0{\cdot}001$) the release of $^{45}Ca^{++}$ from frog sartorius muscle.

$^{45}Ca^{++}$ *Release from Lobster Nerve*

Protoveratrine A (1, 10 or 100 μg./ml.) had no significant effect ($P > 0{\cdot}9$) on release of $^{45}Ca^{++}$ from lobster nerve.

Discussion

Early pharmacological studies on the veratrum alkaloids were made with the mixture known as veratrine. This has actions qualitatively similar to those of the purified ester alkaloids which, in the intact animal, cause reflex hypotension, bradycardia and bradypnoea. This reflex is known as the Bezold-Jarisch reflex and is due to sensitisation of receptors in the left heart, great vessels and lungs[11], which respond by firing at an increased frequency. Similar phenomena can be demonstrated in isolated nerve and muscle and have been observed after changes in the ionic composition of the bathing fluid[2]. Initiation of a nerve volley at the sensory endings is accompanied by an alteration in the permeability of the cell membrane with alterations in the relative concentrations of K^+ and Na^+. There is some evidence that these changes are related to alterations in calcium-binding at the cell surface[12]. Reduction of the concentration of Ca^{++} in the external medium causes repetitive firing in nerve and muscle and an increase in the external concentration of Ca^{++} abolishes the sensitising effects of the veratrum alkaloids[2]. From our results it appears that the influence of protoveratrine A on nerve and muscle differs. No increase in K^+ efflux from protoveratrine A-treated skeletal muscle was shown. This is in contrast to the observations of Rosenblueth[3] and Shanes[4] using nerve but agrees with Harris (personal communication), who used frog muscle and Kahn and Acheson[6], who used erythrocytes. The increased K^+ efflux from cardiac muscle after protoveratrine A may reflect differences in the rate of metabolism, in the mechanism of potassium transfer or in the lability of intracellular potassium and its freedom

to exchange, but it must also be remembered that protoveratrine has a direct action on receptors in the heart. Low concentrations of veratrum alkaloids initiate the Bezold-Jarisch reflex by acting on sensory nerve endings in the coronary artery bed[13]. Most (but not all) of the effluent in our experiments has passed through the coronary circuit. K^+ release may occur more readily at this site and this may explain why the Bezold-Jarisch reflex is elicited by doses of protoveratrine which have little or no effect elsewhere. It is difficult to explain why protoveratrine-treated muscles took up more $^{42}K^+$ than the controls.

Protoveratrine in concentrations which displace $^{45}Ca^{++}$ from muscle also promotes uptake of $^{42}K^+$. Displacement of Ca^{++} may free anionic sites on the cell surface which can bind K^+ in excess of that already present. Gordon and Welsh[5] suggested that veratrine acted by displacing Ca^{++} from the cell membrane, but their evidence was indirect and no measurements of Ca^{++} concentrations were made. Our results on muscle support this theory, but we found no release of Ca^{++} from lobster nerve. Monné[14] has suggested that the polypeptide chains of the surface protein may be linked by divalent Ca^{++}. Making and breaking of this link alters the configuration of the protein surface and may account for changes in permeability often associated with alteration in Ca^{++} concentration. Protoveratrine is a highly hydroxylated molecule[15]; it may form hydrogen bonds with the protein of the cell surface preventing Ca^{++} from performing its normal linking function and so induce changes in permeability.

We believe that our results support the view that protoveratrine and the related veratrum alkaloids interfere with the normal metabolism of Ca^{++} on the cell surface, altering membrane permeability and thus ionic balance which is the factor finally responsible for the characteristic actions of the veratrum alkaloids on nerve and muscle.

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References

1. Krayer and Acheson, *Physiol. Rev.*, 1946, **26**, 383.
2. Goffart and Bacq, *Ergeb. der Physiol.*, 1952, **47**, 555.
3. Rosenblueth, Wills and Hoagland, *Amer. J. Physiol.*, 1941, **133**, 724.
4. Shanes, *Ann. N.Y. Acad. Sci.*, 1952, **55**, 1.
5. Gordon and Welsh, *J. Cell. Comp. Physiol.*, 1948, **31**, 395.
6. Kahn and Acheson, *J. Pharmacol.*, 1955, **115**, 305.
7. Lister, Ph.D. Thesis, University of Glasgow, 1959.
8. Fenn, *Physiol. Rev.*, 1940, **20**, 377.
9. Harris, *J. Physiol.*, 1955, **30**, 23P.
10. Vick and Kahn, *J. Pharmacol.*, 1957, **121**, 389.
11. Heymans and Neil, *Reflexogenic Areas of the Cardiovascular System*, 1958, London, J. & A. Churchill Ltd.
12. Brink, *Pharmacol. Rev.*, 1954, **6**, 243.
13. Dawes, *J. Pharmacol.*, 1947, **89**, 325.
14. Monné, *Advances in Enzymology*, 1948, **8**, 1.
15. Kupchan, Neeman, Ayres, Hensler and Rajagopalan, *Chem. Ind.*, 1958, 1626.

THE EFFECTS OF PROTOVERATRINE ON PLASMA POTASSIUM LEVELS IN THE CAT AND RABBIT

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Protoveratrine has been shown to increase the level of venous plasma potassium (K^+) in intact rabbits and anaesthetised cats. By the use of restricted circulation experiments it has been demonstrated that at therapeutic dose levels protoveratrine causes an increase in the level of K^+ in the plasma of blood from the coronary circulation but not from skeletal muscle. The possible significance of this finding and its relation to the Bezold-Jarisch reflex is discussed.

Purified preparations of the veratrum alkaloids have been used to reduce blood pressure in hypertension without the production of ganglion blockade[1]. The alkaloids act mainly on peripheral sensory receptors[2] and appear to make them more sensitive to their normal stimulus[3,4,5]. In 1939 Bacq[6] showed that veratrine could sensitize frog muscle to the stimulant action of the potassium ion (K^+), and more recent work has shown that this property is also possessed by the pure ester alkaloids[7].

We have made a number of experiments with the purified ester alkaloid protoveratrine to find if the intravenous injection of this alkaloid produces an increase in the K^+ concentration of the blood plasma. Samples of blood were taken from different vascular beds and the plasma K^+ concentration determined.

Materials and Methods

The protoveratrine was a mixture of the hydrochlorides of protoveratrine A and protoveratine B in the proportions of 2:1. It was obtained as a solution containing 0·1 mg./ml. of alkaloids in a 0·6 per cent w/v solution of sodium chloride. The control solutions consisted of 0·9 per cent w/v sodium chloride.

Plasma K^+ of Rabbit Venous Blood

Rabbits of either sex weighing from 2·25 to 5·0 kg. were used. The animals were gently restrained and two 1 ml. samples of blood taken from one marginal ear vein. The appropriate volume of drug solution was adjusted to 1 ml. with 0·9 per cent w/v sodium chloride and injected into the marginal vein of the other ear 10 minutes after taking the first blood samples. Further samples were taken from this ear 2 and 20 minutes after the drug injection.

The blood was collected into graduated centrifuge tubes each containing 100 i.u. of heparin. The heparinised blood was centrifuged in a M.S.E. angle head centrifuge at 3600 r.p.m. for 10 minutes. Three 0·2 ml. samples of plasma were pipetted from each tube, diluted with K^+-free distilled water and the K^+ concentration determined with an E.E.L.

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flame photometer and expressed as m-equiv./litre. Samples showing haemolysis were discarded.

Plasma K^+ Level of Blood from the Cardiopulmonary Region of Spinal Cats

The spinal cords of cats were cut in the cervical region under ether anaesthesia and the brains destroyed. Ninety minutes were allowed to elapse to allow for elimination of the anaesthetic and for ionic equilibration following surgical trauma.

Drugs were injected into a cannula in the external jugular vein and blood samples taken from a siliconed glass cannula in the left common carotid artery. Two 1 ml. blood samples were taken for control purposes before the drug injection and then at 2 and 30 minutes after the injection, because the hypotension produced by intravenous protoveratrine in lightly anaesthetized cats reaches its maximum at 2 minutes and disappears after 30 minutes. Blood samples were collected and plasma K^+ concentrations estimated as described above.

Doses of 10 or 20 μg. of protoveratrine were given and the number of doses administered to each animal limited to three to prevent the development of tachyphylaxis[2].

K^+-Release from Cat Skeletal Muscle

The effects of protoveratrine on the release of K^+ from skeletal muscle were studied in the denervated hind limbs of pentobarbitone-anaesthetised cats. One limb was used to test the effects of the drug and the contralateral limb as a control.

Intra-arterial injections were made into a needle type injection cannula tied into a minor branch of the femoral artery high in the thigh in such a way that the tip of the cannula was at the junction of this branch with the femoral artery. A similar cannula was tied into a branch of the femoral vein at the same level in the limb.

Total doses of 2 or 5 μg. of protoveratrine in a volume of 0·1 ml. were injected into the femoral artery and blood samples of 0·5 ml. removed from the vein 5, 10, 30, 60 and 120 seconds after injection. Simultaneous injection of the control solution into the contralateral leg and collection of blood samples were made for the purpose of comparison.

Arterial blood pressure was recorded from the left common carotid artery by means of a mercury manometer.

K^+-Release from the Heart of Anaesthetised Cats

As a large component of the reflex hypotensive effect of protoveratrine is believed to arise from receptors in the coronary bed[3,5] the effect of injection of small doses of protoveratrine into the left common coronary artery on the K^+ concentration of coronary venous blood plasma was studied.

The technique used was a modification of that described by Dawes[8] for the administration of drugs into the left common coronary artery combined with the technique first described by Morawitz and Zahn[9] for obtaining samples of blood from the coronary sinus.

Cats of either sex weighing from 3·5 to 6·0 kg. were used. They were anaesthetised by intraperitoneal injection of 40 mg./kg. of sodium pentobarbitone. The thorax was opened on the left side between the fourth and fifth ribs and the animal artificially ventilated using a Starling respiration pump. All bleeding points were sealed with electro-cautery. The space between the ribs was enlarged using rib retractors and the heart and great vessels exposed. The left subclavian artery was cleared of connective tissue and the pericardium incised and reflected. The left

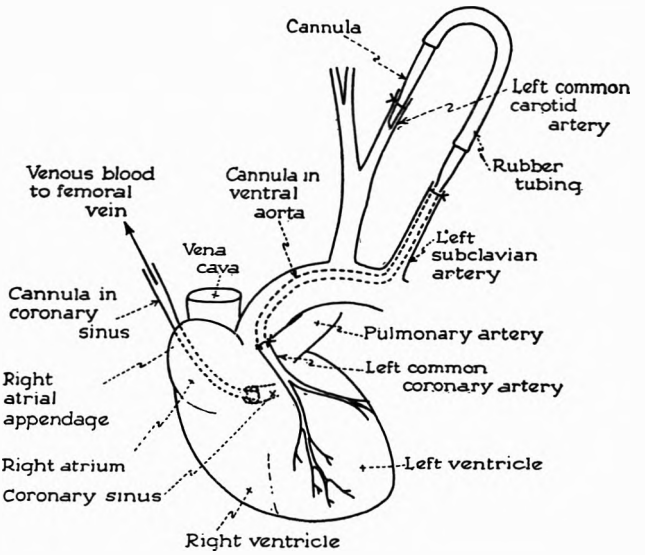

Fig. 1 *Position of cannulae in cat heart.*

common coronary artery was then cleared from adhering fatty tissue and a loose ligature passed round it. The coronary sinus cannula was introduced into the right atrium through an incision in the right atrial appendage, the tip introduced into the coronary sinus and the cannula secured by means of a drawstring suture round the incision in the appendage. A siliconed glass cannula similar to that described by Dawes[8] was introduced into the left subclavian artery and, guided by touch, pushed caudally until its tip was in the region of the lumen of the left common coronary artery. Its distal end was connected by a piece of flexible rubber tubing to a glass venous type cannula in the left common carotid artery. The tip of the cannula was tied into the left coronary artery thus establishing a closed circuit (Fig. 1).

Blood pressure was recorded from the right femoral artery and a continuous E.C.G. recording was taken from lead II using subcutaneously implanted needle electrodes. The record was made and displayed with an Ediswan direct writing pen recorder. After the operation was complete the animal was heparinised and given an intravenous infusion of 15 ml. of normal saline to restore the blood volume. Drugs were introduced

TABLE I

THE EFFECTS OF PROTOVERATRINE ON THE PLASMA K^+ OF INTACT RABBITS

Experiment No.	Sex	Age in months	Wt. in kg.	Dose µg./kg.	Mean K^+ levels of plasma m-equiv./l.			Symptoms
					Before drug	After drug		
						2 min.	20 min.	
1	M	*c.* 24	5	10	4·43	3·76	3·74	No obvious signs of discomfort. Some bradycardia
2	M	5	2·5	10	4·98	5·50	4·95	ditto
3	M	6	2·8	10	3·31	3·83	4·47	ditto
4	F	5	2·8	10	4·46	4·98	4·60	ditto
5	M	5	2·75	20	5·63	5·67	5·63	Slight signs of discomfort, retching
6	M	6	4·5	20	4·60	5·88	4·75	Sneezing, loss of muscular tone, tachypnea, bradycardia
7	M	5	3·1	20	5·10	5·26	5·36	ditto
8	M	3	2·75	20	3·58	5·88	4·60	Sneezing, muscular tremors, attempted vomiting, periods of apnea, bradycardia
9	M	6	2·25	20	3·66	5·26	4·60	Sneezing, retching, salivation, bradycardia
10	F	6	3·0	20	4·56	5·45	5·05	ditto
11	M	6	2·25	30	4·34	4·60	4·35	Attempted vomiting, irregular respiration and apnea, slight convulsions, bradycardia
12	F	3	2·0	30	4·75	16·8	23·1	Spasmodic head movements, attempted vomiting, extension of neck, followed by loss of muscular tone, convulsions and death

TABLE II

THE EFFECT OF PROTOVERATRINE ON THE PLASMA K^+ LEVEL OF CAROTID BLOOD IN SPINAL CATS

			Sampling time in minutes																			
Experiment No.	Sex	Weight in kg.	90	120		150		180		210		240		270	300	330		360		390
1	F	2·5				4·35		4·25	*4·87*	5·33										
2	F	3·5	3·58	4·35						3·84	*3·80*	4·20			6·30	8·40				
3	M	2·75	4·1	5·30				5·30				6·40								
4	F	2·5	4·6	4·25		5·45				4·75	*4·82*	5·20		5·20	6·90	6·15	*6·65*	7·30		
5	F	2·6	4·95	4·75		5·20				5·10	*5·21*	5·37		5·10						
6	F	3·0	2·76			3·50				4·20				3·40	4·20	5·0	*5·75*	3·9	4·3	4·6
7	M	3·25	2·98	3·23				3·33		3·33	3·92	3·94		3·46	3·90	4·8	5·24	4·08		
8	F	3·25	3·40	3·71				3·96		3·71	*3·35*			3·56	4·10	3·46	3·86	3·22	3·76	3·96
9	F	3·2	2·80	2·70	2·80	2·78		3·32		3·20	5·4	4·6		6·1						
10	F	2·0	3·54	3·68		3·42	4·8	3·68												
11	M	2·6	2·62	3·08				3·76		4·23		5·0	5·0	5·0	4·25					

Italic figures = K^+ level 1 minute after the injection of 10 μg. protoveratrine.
Bold figures = K^+ level 1 minute after the injection of 20 μg. protoveratrine.
All K^+ concentrations expressed as m-equiv. of K^+ per litre of plasma.

into the coronary circulation by injection through the rubber tubing joining the two cannulae. Blood drained from the coronary sinus was reintroduced into the right femoral vein.

Doses of 2 or 5 μg. of protoveratrine in 0·1 ml. of solvent or the equivalent volume of control solution were introduced into the coronary circulation. The effects of the drug on the blood pressure and E.C.G. were recorded. The doses of drugs used, produced a fall in blood pressure and a bradycardia readily detected by the E.C.G. Control solutions produced only a transient fall in blood pressure and no detectable bradycardia.

The maximum changes in blood pressure and heart rate occurred from 2 to 5 seconds after injection of the drug and at these times samples of coronary venous blood were taken and analysed for plasma K^+ using the method previously described.

Results

Plasma K^+ Levels in Rabbit Venous Blood

The object of this series of experiments was to determine if any gross changes in plasma K^+ levels occurred after intravenous injection of protoveratrine in doses sufficient to produce a marked fall in blood pressure.

Fairly high doses of protoveratrine (10, 20 or 30 μg./kg.) were used to produce a very marked effect. At the 10 μg./kg. level no obvious toxic symptoms were observed but bradycardia was noticed in two out of the four animals used. With higher doses signs of distress became apparent. The plasma K^+ levels before and after protoveratrine are shown in Table I. Plasma K^+ levels before injection of the drug were within the range given by Spector[10], *i.e.* 2·7 to 5·1 m-equiv./litre, in eleven of the twelve experiments. In these the plasma K^+ level was significantly raised after intravenous injection of protoveratrine ($P = 0{\cdot}05$). The rabbit in experiment No. 12 died showing a high plasma K^+ level before death. This animal was not included in the statistical test for significance. At the lowest doses, the increase in K^+ levels was less marked but occurred in three out of the four experiments.

Plasma K^+ Levels of Blood from the Cardio-Pulmonary Circulation

The K^+ concentration of arterial plasma showed marked variations between experiments but was within the range reported by Cattel and Civin[11]. As the experiment proceeded, the concentration rose. This was expected, as operative trauma has been shown by a number of workers to produce an increase in plasma K^+[11–13].

The injection of protoveratrine into the jugular vein frequently gave rise to bradycardia presumably owing to its direct negative chronotropic effect on the heart[2]. The results from eleven experiments are summarised in Table II.

After the injection of 10 μg. protoveratrine the K^+ level rose in six out of eight and with 20 μg. in seven out of eight experiments. No increase was observed after the injection of 0·2 ml. of control solution.

TABLE III

THE EFFECT OF PROTOVERATRINE ON THE K^+ LEVEL OF VENOUS PLASMA TAKEN FROM THE HIND LIMBS OF CATS

Experiment No.	Test: K^+ level of plasma m-equiv./l.						Control: K^+ level of plasma m-equiv./l.					
	Before drug	No. of seconds after drug					Before control	No. of seconds after control				
		5	10	30	60	120		5	10	30	60	120
For 2 μg. Protoveratrine												
1	4·21	4·23	4·20	4·26	4·18	4·21	4·19	4·22	4·17	4·22	4·20	4·18
2	3·76	3·74	3·80	3·71	3·72	3·78	3·80	3·81	3·78	3·92	3·73	3·87
3	3·98	4·10	3·89	4·00	4·01	4·00	3·96	3·96	4·00	3·89	3·94	3·96
4	3·41	3·36	3·41	3·40	3·39	3·50	3·40	3·41	3·37	3·61	3·54	3·44
	Mean ± SE 3·84 ± 0·31						Mean ± SE 3·86 ± 0·27					
For 5 μg. Protoveratrine												
1	4·23	4·24	4·19	4·26	4·20	4·20	4·21	4·22	4·19	4·29	4·18	4·20
2	3·66	3·68	3·57	3·65	3·70	3·69	3·80	3·81	3·69	3·84	3·75	3·72
3	4·12	4·12	4·20	4·15	4·08	4·10	4·00	4·20	4·22	4·16	4·06	4·04
4	3·50	3·41	3·40	3·57	3·49	3·52	3·51	3·41	3·42	3·61	3·44	3·49
	Mean ± SE 3·87 ± 0·32						Mean ± SE 3·89 ± 0·38					

The results indicate that injection of protoveratrine into the cardiopulmonary region produced an increase in the plasma K^+ concentration. This rise may have been caused by the actual liberation of K^+ or to the drug-induced bradycardia reducing the rate of outflow from the coronary system, thus allowing more K^+ to accumulate in the venous outflow. The K^+ may also have originated in the heart or the lungs.

K^+ from Cat Skeletal Muscle

Intra-arterial injection of protoveratrine in total doses of 2 or 5 μg. produced no muscular twitching and in only two cats out of eight was

TABLE IV

THE EFFECTS OF PROVERATRINE ON THE K^+ CONCONCENTRATION OF BLOOD TAKEN FROM THE CORONARY SINUS

Experiment No.	Total dose of protoveratrine	K^+ concn. in m-equiv./l.		Per cent change
		Control	Drug	
1	2 μg. 2 μg. 5 μg.	1·73 1·73 4·02	1·90 1·78 5·30	+ 9·8 + 2·9 + 32·8
2	5 μg.	1·54	2·19	+ 35·7
3	2 μg. 5 μg.	4·86 4·09	5·11 5·37	+ 15·8 + 30·8
4	2 μg.	3·96	4·60	+ 16·2
5	2 μg.	2·94	3·58	+ 21·7
6	5 μg.	1·20	1·65	+ 37·5

Mean per cent increase in K^+ concentration after 2 μg. protoveratrine = 13·3 $\pm$ 3·2 per cent.
Mean per cent increase in K^+ concentration after 5 μg. protoveratrine = 34·2 $\pm$ 3·3 per cent.

hypotension produced by the drug and when this did occur it was only transient.

The results of eight experiments are summarised in Table III. A statistical comparison of the means for the control and the drug-treated limbs showed that there was no significant difference ($P > 0·9$) between the plasma K^+ concentrations of the venous blood from the two.

K^+-Levels in Coronary Sinus Blood

Because of the technical difficulties involved in this series of experiments the mortality rate was high. In all the successfully executed experiments, the results from which are listed in Table IV, intra-coronary injection of small doses of protoveratrine led to a marked increase in K^+ concentration of the blood collected from the coronary sinus. The onset of bradycardia was taken to indicate that the drug had reached the receptor sites.

No increase in the K^+ concentration of coronary sinus plasma occurred after injection of 0·2 ml. of control solution into the coronary circulation.

It therefore appears that under the conditions of these experiments protoveratrine is capable of increasing K^+ efflux from heart muscle.

Discussion

Protoveratrine is a mixture of two very closely related pharmacologically active steroidal ester alkaloids which are qualitatively identical and differ chemically only in their acid moieties[14].

In addition to the action of protoveratrine on the cardiovascular system, as exemplified by the initiation of the Bezold-Jarisch reflex, it can alter the shape and size of the resting and action potentials of nerve and muscle and sensitize excitable tissue to the stimulant actions of K^+ [2,4].

It has been suggested by various authors that the veratrum alkaloids may affect the ionic balance of nerve and muscle cells causing an increase in extracellular K^+ levels[15,16]. Shanes[15] has demonstrated a greater K^+ loss from frog nerves exposed to veratrine than from the control nerve and using protoveratrine we have demonstrated that this drug can produce an increase in the plasma K^+ levels in intact rabbits and anaesthetized cats. Kahn and Acheson[17] have shown that this effect is not due to the release of K^+ from erythrocytes and as the greatest reservoir of K^+ in the body is the skeletal musculature this was considered a possible source of the increased plasma K^+. Our results show, however, that it is unlikely that this excess K^+ originates from skeletal muscle and that a more likely source appears to be the myocardium.

The amount of K^+ released is very small and we have had to use doses of protoveratrine higher than those used in therapy before any measurable change could be detected in the experiments upon the general circulation. Smaller doses of drug, within the range achieved in therapeutics, have been shown to cause an apparent release of K^+ into the restricted circulation of the heart but not from the skeletal musculature. Vick and Kahn[18] have recently demonstrated a similar increase in K^+ efflux from isolated perfused guinea pig hearts and our results appear to confirm their findings.

It is significant that, of the various types of sensory nerve endings which may be stimulated by protoveratrine, those which appear to be stimulated by the lowest concentration of drug, are situated within the walls of the heart in the area of distribution of the left coronary artery and can initiate the Bezold-Jarisch reflex[3,8]. It is possible, therefore, that the relatively high efflux of K^+ occurring in this region after protoveratrine may preferentially affect these endings and so facilitate initiation of this reflex.

Many factors are known to cause, or to be involved in, the release of intracellular K^+ and this study does not indicate the mechanism by which protoveratrine acts. Work on isolated organs and tissues using more refined techniques is reported elsewhere[19].

Acknowledgements.—Our thanks are due to Messrs. Sandoz Products Ltd. for a generous supply of protoveratrine (Puroverine), to Miss G. F. Halley for technical assistance and Mr. R. Callander for the drawing (Fig. 1).

References

1. Smirk, *High Arterial Pressure*, Blackwell, Oxford, 1957.
2. Krayer and Acheson, *Physiol. Rev.*, 1946, **26**, 383.
3. Heymans and Neil, *Reflexogenic Areas of the Cardiovascular System*, Churchill, London, 1958.

4. Goffart and Bacq, *Ergebn. Physiol.*, 1952, **47**, 555.
5. Paintal, *Quart. J. exp. Physiol.*, 1955, **40**, 348.
6. Bacq, *C.R. Soc. Biol., Paris*, 1939, **130**, 1369.
7. Goutier, *Brit. J. Pharmacol.*, 1950, **5**, 33.
8. Dawes, *J. Pharmacol.*, 1947, **89**, 325.
9. Morawitz and Zahn, *Zbl. Physiol.*, 1912, **26**, 465.
10. Spector, *Handbook of Biological Data*, Saunders, Philadelphia, 1956.
11. Cattel and Civin, *J. biol. Chem.*, 1938, **126**, 633.
12. Danowski and Elkinton, *Pharmacol. Rev.*, 1951, **3**, 42.
13. Scudder, Smith and Drew, *Amer. J. Physiol.*, 1939, **126**, 337.
14. Goodman and Gilman, *The Pharmacological Basis of Therapeutics*, 2nd ed., Macmillan, New York, 1955.
15. Shanes, *Ann. N.Y. Acad. Sci.*, 1952, **55**, 1.
16. Rosenbleuth, Wills and Hoagland, *Amer. J. Physiol.*, 1941, **133**, 724.
17. Kahn and Acheson, *J. Pharmacol.*, 1955, **115**, 305.
18. Vick and Kahn, *ibid.*, 1957, **121**, 389.
19. Lister and Lewis, *J. Pharm. Pharmacol.*, 1959, **11**, Suppl., *176 T.*

After Dr. Lister presented both papers there was a DISCUSSION.

Short Communication

METALLIC CATIONS AND THE ANTIBACTERIAL ACTION OF OXINE

By A. H. Beckett, R. N. Dar and Ann E. Robinson

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The binding of cobaltous and manganous ions by *Staphylococcus aureus* suspensions, alone and in combination with oxine and with iron, has been examined as part of a study of the mechanism of antibacterial action of oxine[1]. The antibacterial properties of oxine solutions containing iron, cobalt and manganese ions, alone or in combination, have been evaluated. These metal ions were chosen because Albert and others have reported the unique action of cobalt in reversing the bacteriostatic and bactericidal actions of iron-oxine solutions against Gram-positive bacteria and that oxine solutions containing cobalt or manganese were much less toxic to *Staph. aureus* than those containing iron (ferrous or ferric), copper or cadmium[2].

The concentrations of metal ions remaining in solution after contact with the bacterial suspensions were determined colorimetrically: total iron was determined as the ferrous-*o*-phenanthroline complex, manganese as the permanganate ion and cobalt as a complex with α-nitroso-β-naphthol 3,6-disulphonic acid (Nitroso R salt). Quantitative recoveries of each of these metal ions were achieved under the conditions obtaining in the biological investigations.

The extent of iron, cobalt and manganese ion binding by *Staph. aureus* suspensions was independent of the contact time between 20 and 60 minutes; the initial metal ion concentrations were sufficient to achieve maximum uptake by bacteria (Fig. 1). Binding was at least 90 per cent complete within 2 minutes.

The curves obtained by plotting the molar concentration of ions bound by *Staph. aureus* suspensions (standardised nephelometrically) against the equilibrium concentration were similar in shape for iron, cobalt and manganese although the maximum uptake of iron and manganese exceeded that for cobalt. Mass Law plots (Rothstein and Hayes[3]) of these results indicate that iron and manganese ions are each bound at two different receptor sites whereas cobalt is bound at only one, thus supporting the previous postulate of binding at an anionic receptor site and a chelating site[1].

An estimation of the number of iron and manganese atoms bound per bacterium is $6{\cdot}5 \times 10^7$, whereas the corresponding figure for cobalt is 4×10^7. The divalent metal ions have similar ionic atomic radii (cobalt 0·72, iron 0·75 and manganese 0·8 Å, neglecting water of hydration) and a common co-ordination number of 6. Although the relative affinities of bivalent cations of the first transition series is $Mn^{++} < Fe^{++} < Co^{++}$

$< Ni^{++} < Cu^{++} > Zn^{++}$[4], the oxidation state of the metal ions on binding at the bacterial surface is uncertain and this order will not necessarily be observed. Experiments with solutions initially containing equimolar proportions of iron and manganese showed that manganese was preferentially bound by *Staph. aureus* suspensions. Further, the total concentration of iron and manganese bound reached a constant value equivalent to the maximum uptake of either iron or manganese alone, thus implying interchangeability at common binding sites. Using solutions containing iron and cobalt, however, the total concentrations of ions bound varies with the relative proportions of the two ions; it may exceed the level of maximum uptake for cobalt alone but does not attain that for iron alone. Cobalt, therefore, appears to compete with iron for one binding site and to prevent normal iron binding at the second; the latter effect might be caused by bound cobalt atoms partially masking adjacent binding sites.

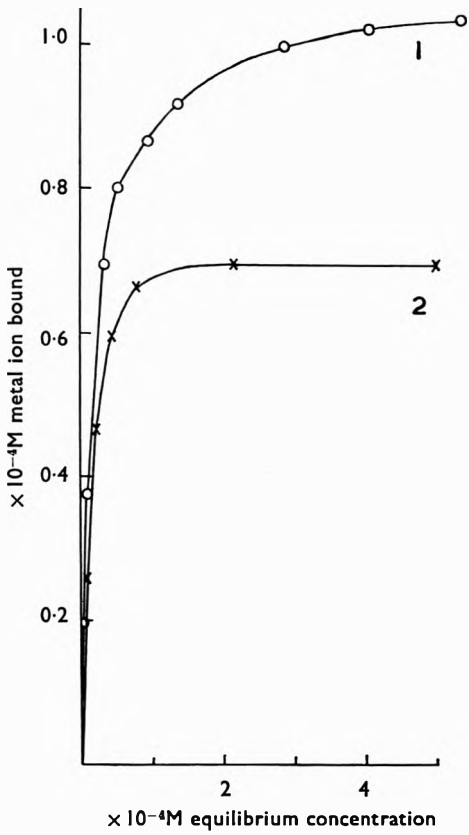

FIG. 1. The uptake of iron (1) and cobalt (2) from their separate solutions by *Staph. aureus* suspensions containing approximately 10^9 organisms/ml. The results for manganese are similar to those for iron.

Slight potentiation of metal binding by *Staph. aureus* was effected by the addition of a small proportion of oxine (1×10^{-5} M) to the contact solutions (molar ratio of oxine : metal ion 1 : 10 or less). The analytical methods were insufficiently sensitive to allow a reduction of the molar ratio of oxine: iron to 1 : 1.

Bactericidal evaluation was carried out under similar conditions to those for the uptake work; the metal ions, *per se*, at maximum concentrations of 1×10^{-4} M for iron, cobalt and manganese were inactive, as was oxine at 1×10^{-5} M. The maximum contact time in all these experiments was 150 minutes. Using solutions containing a constant concentration of oxine (1×10^{-5} M) and varying proportions of iron, optimum bactericidal activity against *Staph. aureus* was attained when the molar ratio of iron : oxine was about 3 : 1; the activity decreased markedly

if the ratio was reduced to 0·5 : 1. Solutions containing equimolar proportions of oxine (1×10^{-5} M) and either cobalt or manganese were devoid of activity. Addition of a 5 mole excess of manganese to an iron-oxine solution (1 : 1 molar ratio, 1×10^{-5} M) did not reduce the bactericidal activity, whereas some loss occurred on substitution of a similar molar proportion of cobalt for the manganese.

Thus, traces of cobalt were less effective in reversing the bactericidal effects of iron-oxine solutions against *Staph. aureus* in this system than in the one used by Albert, who found that $0{\cdot}2 \times 10^{-4}$ M cobalt would abolish the bactericidal action of a solution containing iron ($0{\cdot}2 \times 10^{-4}$ M and oxine ($0{\cdot}4 \times 10^{-4}$ M) against *Staph. aureus*[5]. Variations in the degree of efficiency of cobalt in this rôle may probably be attributed to the differences in the experimental procedures adopted.

References

1. Beckett, Vahora and Robinson, *J. Pharm. Pharmacol*, 1958, **10**, Suppl., *160T*.
2. Rubbo, Albert and Gibson, *Brit. J. exp. Path.*, 1950, **31**, 425.
3. Rothstein and Hayes, *Arch. Biochem. Biophys.*, 1956, **63**, 87.
4. Irving and Williams, *J. chem. Soc.*, 1953, 3192.
5. Albert, in *The Strategy of Chemotherapy*. *Symp. Soc. gen. Microbiol.*, 1958, **8**, 112.

After Dr. Robinson presented the communication there was a DISCUSSION.

A COMPARISON OF THE SPASMOLYTIC EFFECTS OF TWO PHENYLETHYLAMINES AND SOME OBSERVATIONS ON MORPHINE-LIKE ACTIVITY

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Contraction of isolated guinea pig ileum caused by 5-hydroxytryptamine, acetylcholine, histamine and barium was prevented by the isopropyl and cyclohexyl ethers of 1-(*p*-hydroxyphenyl)ethylamine at similar concentrations to those found in rat tissues after injection. Inhibition due to the isopropyl ether was immediate and readily washed out. It did not antagonise substance P. Inhibition by the cyclohexyl ether was persistent and needed a short incubation period for full potency to develop. It antagonised substance P. The results are introduced against a background of a broad speculation on the nature of morphine-like activity. Ammonium ion partially inhibited 5-hydroxytryptamine at a concentration similar to that found *in vivo* during convulsions.

The sum total of morphine's effects in the animal seems to be a good example of Gunn's pharmacological syndrome[1]. It could be a physiological syndrome in the manner that the adaptation syndrome or sympathomimesis can be so regarded. It is not difficult to portray the morphine syndrome as an antithesis and subsidiary to sympathomimesis. It is such as might be expected of a non-specific summation of changes that occur after receipt of a variety of noxious stimuli, changes that severally can assist the maintenance of passive states. These are characterised in the main by immobility whereas sympathomimesis fits the animal for the mobility of fight or flight. Well defined passive states include hibernation, feigned death and the immobility of "freezing". They are initiated by noxious stimuli that for reasons such as a lack of orientation in space or insuperability, are difficult to avoid. When fight or flight is impracticable, protection may often be conferred by immobility. This speculative and ramifying topic cannot be elaborated here but two points need emphasis. No drug randomly distributed by blood can be expected to mimic faithfully the various protective reactions that arise after permeation of sensory information through the filter of nervous integration. At best there can be a non-specific and qualitative summation of various reactions. Morphine need not induce a passive state, just as adrenaline need not cause fight or flight.

On the above grounds and by analogy with sympathomimesis, it was surmised that morphine and its substitutes could either imitate, release, inhibit or modify tissue response to, some substance concerned with the maintenance of state and, by hypothesis, associated with a diffuse efferent neural network. So far, only adrenaline, and possibly cortisol, have been recognised as natural agents that help to maintain a change in state in the body as a whole, though local hormones[2] appear to control the state, such

as tone, of individual organs. "Inhibitory transmittors" have been postulated from time to time to explain experimental findings but it was convenient in this instance to consider some known humoural substances first. Of these, 5-hydroxytryptamine and substance P have most interest relative to morphine. The richest tissue sources of both are the autonomic brain and intestine, tissues that are probably directly influenced by the drug.

The effects of substance P in the whole animal tend to run counter to those of morphine. It rendered mice hyperalgesic and antagonised morphine analgesia[3]. The animals were sedated[3,4]. It induced tachypnoea and hyperpnoea[5]. Conversely, like morphine, it was a transient depressor agent by vasodilation and increased intestinal tone[6]. The distribution in tissues has suggested an association with the first sensory neurone[7]. 5-Hydroxytryptamine was a potent pain producing agent[8]. Morphine antagonised 5-hydroxytryptamine spasm in guinea pig ileum[9,10] and caused a lasting depression of the indole's effect on the superior cervical ganglion[11]. Morphine released the indole from rat tissues[12]. The depressant effect of 5-hydroxytryptamine on brain was antagonised by morphine[13], whereas the psychomotor effects of morphine were antagonised by reserpine[14,15].

A simple experimental approach was to compare the spasmolytic effect of the isopropyl and cyclohexyl ethers of 1-(*p*-hydroxyphenyl)-ethylamine on the isolated intestine. This tissue may provide a model of certain hypothalamic processes[16] with the advantage that the end result of any action is observable as a muscle movement rather than a synaptic discharge. Agents that influence intestinal activity often influence analgesia. Papaverine may potentiate morphine analgesia[17]. Antihistamines[18,19] have been described as analgesic. Anticholinergic compounds potentiated analgesia[20] and morphine reduced acetylcholine output by isolated intestine[16,21]. There are numerous descriptions of the analgesic effect of adrenaline[22].

During several years the above-mentioned amino ethers have been shown to behave almost identically in a variety of experimental situations but only the cyclohexyl ether mimicked morphine in the rat[23]. The mimicry extended to antagonism of analgesia by the *N*-allyl ether[24]. It seemed that any notable difference between the two ethers could indicate an approach to the mechanism of morphine-likeness. Notable differences were seen in the influence of high concentrations (4×10^{-2}M) on the intestine such as occurs by intraperitoneal injection. The cyclohexyl ether abolished intestinal movement in rats and caused a petechial haemorrhage with a serous exudate containing cholesterol and protein. After several daily doses there was a flaccid distension of the intestine. Morphine in single doses enhances the tone of the muscle but distension has been described in morphine addicts[25]. These effects were never seen in many experiments with the isopropyl ether. As for morphine, some of the effects seem reminiscent of histamine release but, using the Evans blue permeability test, no difference could be found in the ability of the two amines to release histamine from rat skin.

This paper is concerned with a comparison of the spasmolytic effect of the two ethers against the common spasmogenic agents used in investigating drug effects on the intestine.

Methods and Materials

Substance P was extracted either from fresh horse intestine (method of Pernow[26] to the alcohol precipitation stage) or was obtained in solution from the pooled intestines of six rats by a similar method. 2·5 kg. of horse tissue gave 2·04 g. of a buff powder containing 30 per cent ammonium sulphate. It caused contraction of guinea pig ileum at 4 to 20 μg./ml. but appeared to contain 5-hydroxytryptamine since the contraction was partially antagonised by tryptamine. Dialysis and short alkaline but not acid hydrolysis, destroyed the activity but tryptic digestion was only partially effective. The rat preparation contained no 5-hydroxytryptamine and was active at 2 μl./ml. For assay, a bath of 5 ml. capacity was used at 37°. When substance P and 5-hydroxytryptamine were the spasmogenic agents the bathing Tyrode contained atropine and mepyramine (10^{-6})[26].

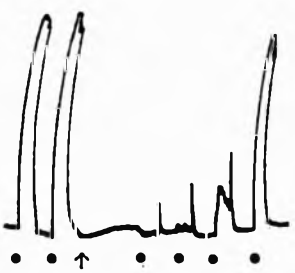

Fig. 1. Inhibition of contractile effect of substance P on guinea pig ileum by 1-(*p*-cyclohexyloxyphenyl)-ethylamine. 20 μl. of substance P solution prepared from rat intestine added at ●. 2×10^{-5}M amine added at the arrow, followed by 3 min. incubation.

Substance P was assayed in tissues after injection of drugs into rats. The tissue was extracted with hot hydrochloric acid at pH 4 for 2 minutes. Substance P was precipitated by saturation with ammonium sulphate and the precipitate extracted with 90 per cent ethanol. The extract was dried at 0°/5 mm. and the residue preserved at −10° until required. About 200 mg. of ammonium sulphate contaminated the extract, sufficient to give a concentration of about 10^{-5}M in the bath. Recovery of known amounts of substance P activity averaged 55 ± 6 per cent.

Fig. 2. Prolonged inhibition of contractile effect of 5-hydroxytryptamine on guinea pig ileum by 1-(*p*-cyclohexyloxyphenyl)-ethylamine. 5 μg. 5-hydroxytryptamine creatinine sulphate added at ●. 2×10^{-5}M amine added at the arrow, followed by 3 min. incubation.

Results

Attempts to reduce the substance P content of rat intestine and brain by drugs were unsuccessful. Morphine (10 mg./kg. 30 minutes before killing), reserpine (3 mg./kg. 24 hours before killing) and thyroxine (6 daily doses of 50 μg.) all intraperitoneally, were ineffective.

Both the isopropyl and cyclohexyl ethers of 1-(*p*-hydroxyphenyl)-ethylamine inhibited the contraction of guinea pig ileum caused by acetylcholine (0·1 μg.), histamine phosphate (1 μg.), 5-hydroxytryptamine (2 μg.) and barium chloride (0·2 mg.). The amounts quoted refer to a particular experiment where the doses were adjusted to give equal heights of contraction in the one piece of tissue. There was little to distinguish the immediate inhibitory effect of 2×10^{-5}M concentration of either ether. Inhibition by the isopropyl ether was removed by a single wash and it did not antagonise substance P. Inhibition by the cyclohexyl ether was not always seen immediately after addition but, allowing 3 minutes' contact with the tissue, followed by a single washing, there developed a strong antagonism of the above agents. Substance P was also antagonised (Fig. 1). Removal of the inhibition needed up to twelve washes (Fig. 2). By allowing the inhibition to develop, an effect could be shown against substance P at 2×10^{-6}M. Morphine did not inhibit substance P. A range of phenylethylamines, including ephedrine, hordenine, amphetamine, adrenaline, noradrenaline, mescaline and tryptamine, and a variety of aliphatic amines, all failed to antagonise substance P. It was notable that ammonium ion could partially antagonise 5-hydroxytryptamine at $1–2 \times 10^{-3}$M (Fig. 3). Concentrations of this order (0·6 μM/g.) have been found in brain during convulsions[27].

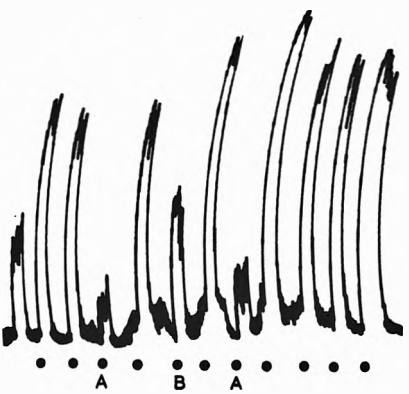

FIG. 3. Inhibition of contractile effect of 5-hydroxytryptamine on guinea pig ileum by ammonium ion. 4 μg. 5-hydroxytryptamine creatinine sulphate added at ●. 2×10^{-3}M ammonium ion added at A. 10^{-3}M ammonium ion added at B.

Discussion

The spasmolytic effects of the isopropyl and cyclohexyl ethers of 1-(*p*-hydroxyphenyl)ethylamine, while non-specific and virtually alike in immediate potency, appear to differ insofar as the cyclohexyl ether needed a period of incubation for its full activity to develop. The inhibition was then persistent. Its ability to inhibit substance P at concentrations as low as 2×10^{-6}M constitutes a distinct difference from the isopropyl ether and may contribute to its effects in the whole animal since concentrations up to 0·3 μmoles/g. (3×10^{-4}M) have been found in rat brain after analgesic doses[28]. Inhibitors of substance P have not been described and

the result suggests that its morphine-like character may arise from this property. Zetler has recently speculated on the role of substance P in morphine analgesia. If the supposition is correct, the mode of analgesic action must differ in detail from that of morphine, which fails to antagonise substance P. It seems possible that a solution to the problem of morphine-like activity could be found in neurohumoural interplay, especially regarding 5-hydroxytryptamine and substance P. Thus it is of interest that the action of morphine on the intestine declines from the duodenum towards the colon and so also does the substance P content[29]. It seems reasonable that the quiescence of the intestine after intraperitoneal injection of the cyclohexyl ether is a consequence of the persistent antagonism to substance P and 5-hydroxytryptamine.

References

1. Gunn, *Arch. int. Pharmacodyn.*, 1935, **50**, 379.
2. Burn, *Physiol. Rev.*, 1950, **30**, 177.
3. Zetler, *Arch. exp. Path. Pharmak.*, 1956, **228**, 573.
4. Stern and Hukovic, *Naturwissenschaften*, 1957, **44**, 517.
5. Euler and Pernow, *Nature, Lond.*, 1954, **174**, 184.
6. Gernandt, *Acta physiol. scand.*, 1942, **3**, 270.
7. Lembeck, *Arch. exp. Path. Pharmak.*, 1953, **219**, 197.
8. Armstrong, Jepson, Keele and Stewart, *J. Physiol.*, 1957, **135**, 350.
9. Kosterlitz and Robinson, *ibid.*, 1955, **129**, 18P.
10. Gaddum and Picarelli, *Brit. J. Pharmacol.*, 1957, **12**, 323.
11. Trendelenberg, *ibid.*, 1957, **12**, 79.
12. Bhattacharya and Lewis, *ibid.*, 1956, **11**, 202.
13. Gaddum and Vogt, *ibid.*, 1956, **11**, 175.
14. Tripod, Bein and Meier, *Arch. int. Pharmacodyn.*, 1954, **96**, 406.
15. Loewe, *ibid.*, 1956, **108**, 453.
16. Paton, *Brit. J. Pharmacol.*, 1957, **11**, 119.
17. Beecher, Gravenstein, Macris and Reichter, *Fed. Proc.*, 1958, **17**, 348.
18. Jacob, Ambrus and Ambrus, *Ann. Inst. Pasteur*, 1951, **81**, 281.
19. Hougs and Skouby, *Acta pharm. tox. Kbh.*, 1957, **13**, 405.
20. Christensen and Gross, *J. Amer. med. Ass.*, 1948, **137**, 594.
21. Schaumann, *Brit. J. Pharmacol.*, 1957, **12**, 115.
22. Ivy, Goetzl, Harris and Burrill, *Quart. Bull. Northw. Univ. med. Sch.*, 1944, **18**, 298.
23. McCoubrey, *Brit. J. Pharmacol.*, 1953, **8**, 22.
24. McCoubrey, *ibid.*, 1954, **9**, 289.
25. McCoubrey, *Lancet*, 1952, **1**, 91.
26. Pernow, *Acta physiol. scand.*, 1953, **29**, Suppl. 105, 1.
27. Dawson and Richter, *J. biol. Chem.*, 1948, **176**, 1199.
28. Brierley and McCoubrey, *Brit. J. Pharmacol.*, 1953, **8**, 366.
29. Pernow, *Polypeptides which Stimulate Plain Muscle*, ed. Gaddum, Livingstone, 1955, p. 28.

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

SOME EFFECTS OF HYDRALLAZINE ON BLOOD IRON AND AN IRON-CONTAINING ENZYME SYSTEM

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A hydrallazine-iron chelation complex has been prepared and shown to have a m.p. of 210°, and no ultra-violet absorption peak at 265 mμ. It has no hypotensive properties and does not antagonise the pressor response to adrenaline in the rat. At high dose levels hydrallazine inhibits catalase activity *in vitro* but not *in vivo*. The iron catalysed oxidation of cysteine to cystine is also inhibited by hydrallazine. Hydrallazine at high dose levels has been shown to cause haemolysis *in vivo* and *in vitro*. A consistent effect upon iron-excretion could not be shown.

Schroeder and Perry[1] found that compounds capable of forming complexes with metals could sometimes lower the blood pressure of renal-hypertensive rats but not of the normotensive animal. They suggested that these compounds and the weak metal complexes formed, combined *in vivo* with metal ions and so caused a fall in blood pressure. Fallab and Erlenmeyer[2] have studied complex formation between hydrallazine, Fe^{2+}, Zn^{2+} and Co^{2+} but did not prepare an hydrallazine-iron chelate. We have therefore investigated the ability of hydrallazine to combine with iron; to inhibit an iron-containing enzyme system and the iron-catalysed oxidation of cysteine to cystine.

Amongst the side effects of prolonged use of hydrallazine is anaemia. Some hydrazine derivatives, for example phenylhydrazine, are known to cause haemolysis and since hydrallazine has a reactive hydrazine group we have investigated this possibility.

Methods

Preparation of Hydrallazine Chelate

A 2 per cent w/v solution of hydrallazine hydrochloride was added to a 10 per cent w/v solution of ferric chloride hexahydrate. A grey precipitate sparingly soluble in water was formed and was recrystallised from absolute methanol until the melting point was constant at 210°. A 0·02 mg./100 ml. solution of hydrallazine in ethanol has one absorption peak below 210 mμ and a second peak between 255 and 268 mμ, while the hydrallazine-iron complex, 0·072 mg./100 ml. in ethanol, did not display the second peak. The chelate was micro-analysed for nitrogen.

Pharmacological Properties of the Hydrallazine-iron Chelate

A 5 mg./ml. solution of the hydrallazine-iron chelate was prepared in propylene glycol, and the effects of this solution were compared with those of a 5 mg./ml. aqueous solution of hydrallazine hydrochloride and propylene glycol itself. Rats of either sex weighing between 300 and

* Squibb Fellow.

400 g. were anaesthetised by intraperitoneal injection of 0·7 ml./100 g. body weight of a 25 per cent w/v solution of urethane. Drug solutions were injected into the cannulated jugular vein and blood pressure recorded from the common carotid artery.

Effects upon Catalase Activity

Crystalline catalase prepared by the Sigma Chemical Co., was used. Catalase activity *in vitro* and *in vivo* was estimated by the method of Euler and Josephson[3]. In *in vivo* experiments rats of either sex weighing between 150 and 200 g. were injected intraperitoneally with 5 mg./100 g. body weight of hydrallazine hydrochloride and killed after 3 hours. The livers were rapidly removed, weighed and homogenised in ice-cold glass distilled water using a Potter-Elvehjem homogeniser. The homogenate was made up to a volume corresponding to 10 ml./g. of liver and a 1 ml. aliquot of this diluted 10 times with ice-cold distilled water. For estimation of catalyse activity 0·2 to 0·4 ml. of this dilute suspension was added to 50 ml. of 0·01N H_2O_2. Controls were done with normal saline. In both *in vitro* and *in vivo* experiments velocity constants were calculated and plotted against time.

Effects upon Oxidation of Cysteine

Oxidation of cysteine to cystine is catalysed by trace metals and iron and copper are especially effective. The iron-catalysed reaction was studied by the Warburg "direct" method. The main chamber contained 1·9 ml. borate buffer at pH 7 together with 0·4 ml. of a 15 mg./ml. cysteine solution. The side-arm contained 0·1 ml. of $3{\cdot}3 \times 10^{-3}$M ferric chloride solution and 0·6 ml. of water. In experiments with hydrallazine the water was replaced by 0·6 ml. of hydrallazine solution to give final flask concentrations after tipping of 0·01, 0·05, 0·10, 0·50, 1·00 or 2·00 mg./ml. The temperature was $24 \pm 0{\cdot}1°$. After an equilibration period of 10 minutes the contents of the side-arm were tipped into the main chamber and readings taken at 5 minute intervals for 30 minutes.

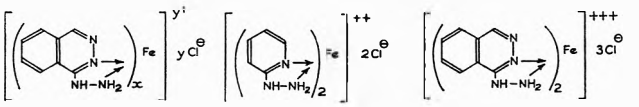
Effects upon Red Blood Corpuscles

For *in vivo* studies rabbits weighing 2·5 to 3·0 kg. were injected intravenously with a solution of neutral $^{59}FeCl_3$ containing 2 μc. of radioactivity. Radioactive iron was obtained as a solution of $^{59}FeCl_3$ containing 20 μc. per 10 ml. Blood samples were collected into heparinised bottles after 24 hours and then on subsequent days for about 30 days. Blood was not collected from the injected ear to avoid contamination from adherent ^{59}Fe. One ml. samples of blood were made up to 10 ml. with water and counted on an Ekco scintillation counter. No attempt was made to separate plasma from corpuscles since almost all of the activity goes into the cells. When the activity had become stable (7 to 9 days) hydrallazine (40 mg./kg.) was injected subcutaneously and blood samples collected 24 hours after the injection and counted. The same procedure was repeated on three subsequent days making a total dose of 160 mg./kg. over a 4 day period. The controls received saline. In some experiments

haemoglobin concentrations and haematocrit values were estimated. Blood samples were collected at intervals for about 21 days after the last injection of hydrallazine. Radioactivity was plotted against time after correcting for decay.

Effects upon Iron Excretion

Rats of either sex weighing about 150 g. were injected intraperitoneally with 2 μc. of ^{59}Fe in the form of neutral $^{59}FeCl_3$ solution. The urine and faeces were collected after 24 hours, combined, partly dried at 100° and



a b c

FIG. 1.

then transferred to a muffle furnace and ashed at 700° for 18 hours. The ash was taken up in dilute hydrochloric acid and adjusted to 5 ml. It was not completely soluble so that the samples were counted in a well-type scintillation counter. Two groups of 4 animals were used. Two in each group served as controls and 2 as tests. Excretion of ^{59}Fe was maximum on the third or fourth day; it then fell and reached a plateau. Hydrallazine

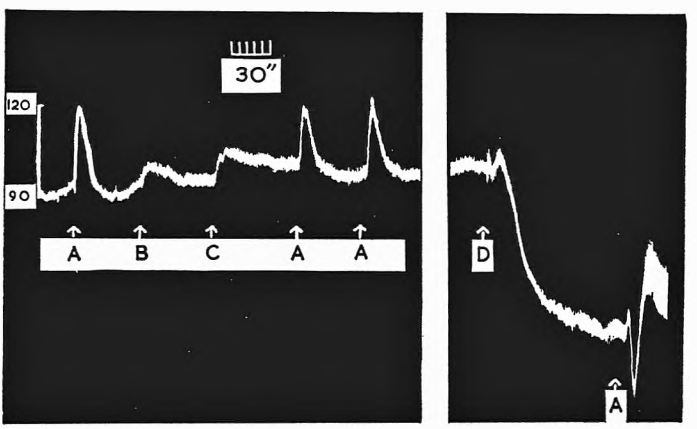


FIG. 2. Rat. Urethane anaesthesia. Blood pressure recorded from common carotid artery and drugs injected *via* the jugular vein.
At A, adrenaline, 0·5 μg./kg.
B, propylene glycol, 0·1 ml./kg.
C, hydrallazine-iron complex, 0·50 mg./kg.
D, hydrallazine, 0·25 mg./kg.

hydrochloride (2·5 mg./100 g.) was injected intraperitoneally on 4 successive days. The urine and faeces were collected, ashed and counted as above.

RESULTS

Hydrallazine Chelates

Certain diamines, for example ethylenediamine, are capable of forming metal chelates. In these the bonds are formed by electron donation and the number of groups associated with the metal is known as the co-ordination number. Iron is known to form chelates and it seems probable that it is forming one with hydrallazine. The five membered ring shown in the general formula (Fig. 1*a*) is theoretically the most stable structure and a similar structure is known in the case of the iron-β-pyridylhydrazine complex (Fig. 1*b*). The analyses obtained for N gave figures of from 22·1 to 23·0 per cent. On the basis of these figures the more probable structure is the one in which the co-ordination number of iron is 4 (Fig. 1*c*). It is probable, however, that we obtained a mixture.

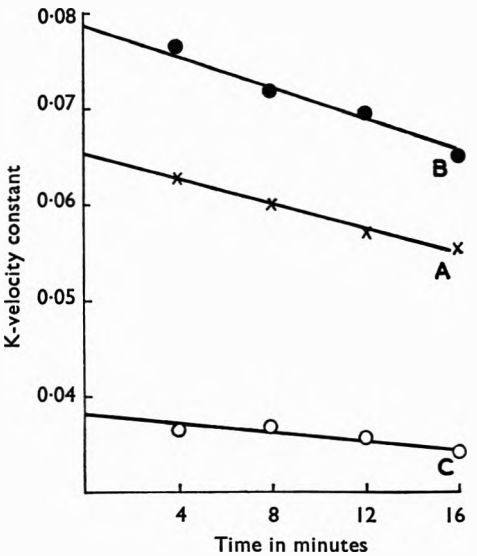


FIG. 3. Effect of hydrallazine on *in vitro* catalase activity.

A, control.
B, hydrallazine, 0·10 mg./ml.
C, „ 10·0 „

Pharmacological Properties of the Hydrallazine-iron Chelate

Hydrallazine causes a marked fall in the blood pressure of the anaesthetised rat and antagonises the pressor effects of adrenaline. The chelate caused no fall in blood pressure but induced a small rise, probably due to the propylene glycol solvent. It did not antagonise the pressor response to adrenaline. Ferric chloride was found to be inert in these respects (Fig. 2).

TABLE I

EFFECT OF HYDRALLAZINE ON IRON-CATALYSED CYSTEINE OXIDATION
Oxygen uptake in μl. ± S.E.

	Concentration μg./ml.	Number of observations	Incubation time (minutes)		
			10	20	30
Hydrallazine ..	10	6	136 (± 6·08)	156 (± 6·24)	152 (± 5·29)
„ ..	50	6	128 (± 10·44)	154 (± 8·06)	153 (± 7·21)
„ ..	100	6	128 (± 8·18)	159 (± 6·16)	154 (± 5·65)
„ ..	500	6	10 (± 0·85)	9 (± 0·87)	7 (± 1·1)
„ ..	1,000	6	12 (± 1·73)	12 (± 2·44)	14 (± 3·31)
„ ..	2,000	3	9·6 (± 1·30)	8·5 (± 2·5)	8·3 (± 7·2)
Control	—	29	132 (± 2·6)	152 (± 2·75)	152 (± 2·91)

Effects upon Catalase Activity

Hydrallazine (0·1 and 1·0 mg./ml.) had little or no effect upon catalase activity *in vitro* but 10 mg./ml. caused about 50 per cent inhibition (Fig. 3). A single dose of 5 mg./100 g. of hydrallazine hydrochloride in rats did not reduce the catalase activity of the liver 3 hours after injection.

Effects upon Oxidation of Cysteine

The results are shown in Table I. Cysteine itself was slightly oxidised at pH 7. Hydrallazine caused a slight oxidation of cysteine. The iron-catalysed oxidation was significantly inhibited ($P < 0.001$) by hydrallazine hydrochloride (0·5, 1·0 or 2·0 mg./ml.), lower concentrations (e.g. 0·1 mg./ml.) had no effect.

Effects upon Red Blood Corpuscles

For these experiments a total of 7 animals of both sexes were used, two of these were controls. The results of a typical experiment are shown in Figure 4. Iron is slowly incorporated into the red corpuscles; the maximum blood radioactivity is reached in 7 to 9 days and this level, once reached, remains constant over the period of the observations. The radioactivity of the blood remained unchanged for 24 to 48 hours after the first or second injection of hydrallazine, but from the third day onwards the activity began to fall. This fall continued on the fourth and subsequent days even though hydrallazine injections were stopped. Activity returned to normal in about 15 days. Four out of five test animals showed these effects but one animal showed no changes in blood radioactivity—treatment being stopped because it had convulsions. In one animal in which haemoglobin and haematocrit estimations were made both showed a fall after hydrallazine, haemoglobin falling from 13·16 to 10·86 per 100 ml. and the haematocrit from 40 to 33. About 16 days after stopping hydrallazine treatment these values returned to normal.

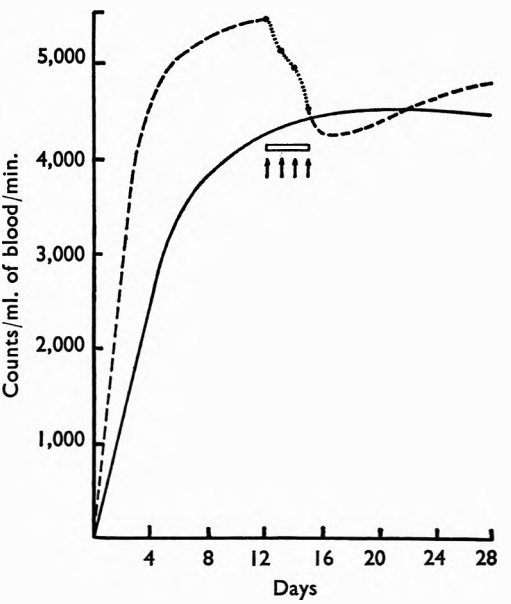


FIG. 4. Effect of hydrallazine on red blood corpuscles. Thick and dotted lines indicate control and test animals respectively. Arrows indicate administration of hydrallazine (40 mg./kg.) to the test animal.

Effects upon Iron Excretion

The counts excreted were plotted against time in days. The number of counts excreted in the urine and faeces increased in 2 animals, 2 others showing no change.

Discussion

The iron-hydrallazine chelate has no hypotensive action in normal rats and does not antagonise the pressor response to injected adrenaline. It has thus lost two of hydrallazine's more important pharmacological properties. It has been reported[4,5] that when hydrallazine is incubated with sera, proteins or polypeptides it loses much of its hypotensive effect. When incubated with an ultrafiltrate of serum there is less loss of activity indicating that the presence of the serum colloids is important. It has also been observed that serum which has first been heated to 56° still causes loss of activity so that an enzyme-catalysed reaction is not likely[3,4]. Hydrallazine is also inactivated by incubation with ground arteries. When it combines with iron, hydrallazine loses its hypotensive activity and the ability to antagonise the pressor response to adrenaline, probably owing to the formation of a stable ring compound. There is no detailed evidence that hydrallazine acts in this way with protein but information on the extent to which it is bound and inactivated by plasma and serum proteins would be valuable.

Hydrallazine inhibits catalase *in vitro* only at very high concentrations; lower concentrations are inactive. Liver catalase activity *in vivo* was not reduced by even the large doses used but the iron-catalysed oxidation of cysteine to cystine was inhibited. This effect may be due to chelation of iron by hydrallazine.

Hydrallazine appears to cause some haemolysis *in vivo* and when a blood sample was incubated at 37° with hydrallazine haemolysis was seen. In rabbits high doses cause a sharp decline in blood radioactivity which rises to normal levels within a few days. This indicates that the iron liberated from the haemolysed corpuscles is apparently used again in the formation of new cells and there is no evidence of inhibition of haemoglobin-synthesis. The results obtained from iron excretion studies do not enable us to draw any conclusions.

Our results do not support the view that hydrallazine acts as an anti-hypertensive by virtue of forming metal chelates. It must be borne in mind that hydrallazine lowers the blood pressure of normotensive animals. The effect of hydrallazine on the red blood corpuscles may explain the anaemias associated with the prolonged clinical use of this drug, especially at high dose levels. Some of the other side effects of hydrallazine may be due to inhibition of cellular oxidation mechanisms.

Acknowledgements. We thank Dr. C. Dale Falconer of Ciba Laboratories Ltd.. for supplies of hydrallazine hydrochloride, Dr. A. H, Beckett, Dr. J. A. Blain, Dr. M. Martin-Smith, Mr. S. Reid and Dr. M. M. Bluhm for help and advice, Miss Sheena MacPhee and Mr. Adam Ritchie for technical assistance and Mr. R. Callander for the drawings.

References

1. Schroeder and Perry, *J. Lab. clin. Med.*, 1955, **46**, 416.
2. Fallab and Erlenmeyer, *Helv. chim. Acta*, 1957, **40**, 369.
3. Euler and Josephson, *Ann. Chem.*, 1927, **452**, 158.
4. Tripod and Meier, *Helv. Physiol. Acta*, 1954, **12**, C.33.
5. Tripod, *Hypotensive Drugs*, Pergamon Press, London, 1956.

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

PHYSICO-CHEMICAL STUDIES OF ASPIRIN WITH GLYCINE

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The relative solubility and rate of dissolution of aspirin in water and glycine solution have been measured. A technique involving a mathematical examination of the front profile of chromatograms has been used to study the extent of the adsorption of glycine from aqueous solution on aspirin. Aspirin is more soluble and more rapidly dissolved in glycine solution than water, and glycine is found to adsorb, in significant amounts, on aspirin crystals. The findings are discussed in an attempt to explain, in physico-chemical terms, differences in taste and adhesion to the oral mucosa that are discernible when aspirin tablets compounded with or without glycine are savoured. Attention is drawn to certain aspects of the results which have a bearing on the transport of aspirin.

Compound tablets of aspirin with glycine are now an accepted form for the administration of aspirin for rheumatic conditions when plain aspirin cannot be tolerated. An editorial in the British Medical Journal[1] draws attention to this method of aspirin therapy, commenting that the physical association of the components is such that they quickly disperse in the mouth even though not taken with a draught of water. No trace of their presence or of any irritant action can be observed gastroscopically[2] and indigestion from their use has not been recorded in fifty consecutive cases. Vining and Kersley[3], examining an aspirin and glycine preparation (Paynocil), found that when this preparation was administered to rheumatic patients intolerant of aspirin, doses of 20 to 100 grains daily could be tolerated without gastric side-effects.

It is a discernible fact that should an aspirin tablet disintegrate in the mouth then the drug can be tasted and the crystalline particles may adhere to the mucosae. If, however, aspirin is compounded with glycine in such a manner that the tablet disintegrates in the mouth within a few seconds, aspirin can no longer be tasted and the particles do not adhere to the mucosae. These observations must be capable of explanation in more precise physical terms and three lines of argument suggest themselves.

The interrelationship of taste mechanism and chemical structure may partly account for the physiological differences in the behaviour of aspirin when compared with aspirin-glycine.

The solubility and rate of dissolution of aspirin in glycine solution may differ from that of aspirin in water.

The formation of adsorbed films of glycine on the aspirin crystals may modify the interaction between aspirin and the protein surfaces of the mucosa.

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The first possibility is conjectural and difficult to expose to experiment. The physico-chemical mechanisms of the other two possible modes of action are readily susceptible to measurement and this paper presents the results of such measurements.

Rates of Dissolution of Aspirin in Water and Glycine Solution

Experimental

Aspirin dissolution has already been reported[4] but this did not include dissolution in glycine solutions.

The aspirin used in this work was Monsanto "Aspirgran"; the glycine was a pure recrystallised material supplied by C. L. Bencard Limited; both materials had a particle size range within the limits 18 to 100 mesh.

Two lots of 500 mg. of aspirin were accurately weighed into a pair of

TABLE I

Comparative rates of dissolution of aspirin in (a) water, (b) 0·25 per cent aqueous glycine solution

t min.	A 20° $E^{1\text{ cm.}}_{225\cdot5\ m\mu}$	A 20° Concentration mg./100 ml.	B 20° $E^{1\text{ cm.}}_{225\cdot5\ m\mu}$	B 20° Concentration mg./100 ml.
2	0·398	83	0·502	105
4	0·620	129	0·777	162
6	0·794	165	0·952	198
8	0·947	197	1·104	230
10	1·002	209	1·202	250
20	1·300	271	1·528	318
30	1·386	289	1·634	340
45	1·425	296	1·685	351
60	1·451	302	1·719	358
90	1·499	312	1·768	368
	A 26°		B 26°	
2	0·997	207	1·191	248
4	1·173	244	1·467	306
6	1·320	275	1·604	334
8	1·417	295	1·706	355
10	1·521	317	1·754	365
20	1·696	353	1·967	410
30	1·828	381	2·057	429
45	1·838	383	2·145	447
60	1·855	386	2·155	449
90	1·887	393	2·176	453

matching 250 ml. Quickfit conical flasks clamped in a Microid flask shaker. At zero time 100 ml. of distilled water was added to one and 100 ml. of 0·25 per cent glycine solution to the other and the shaker started. One ml. samples were withdrawn at timed intervals from each flask and run into 95 ml. of absolute ethanol in 100 ml. graduated flasks, making up to the mark with ethanol. This dilution in ethanol minimised the hydrolysis of the aspirin during the period between withdrawal of the sample and reading the extinction of the solution in a Hilger Uvispek ultra-violet spectrophotometer. The diluted samples were read as quickly as possible against the appropriate blank solution in 1 cm. or 0·5 cm. quartz cells at 225·5 mμ. The E (1 per cent, 1 cm.) for aspirin has previously been found to be at a maximum at this wavelength with a value of 480 in ethanol. The presence of the 0·0025 per cent of glycine

in the final dilution read in the spectrophotometer had no effect on the extinction of the aspirin that could be differentiated from normal experimental error.

The dissolution experiments were made at laboratory air temperature without any attempt at thermostatic control. Repeat runs were made on different days; for one set of experiments the temperature of the two solutions was 20°; on the second occasion the temperature was 26°. The results of these experiments are shown in Table I. From these it can be seen that the solubility of aspirin in 0·25 per cent aqueous glycine is some 15 to 20 per cent greater than in water at laboratory temperatures. The rates of dissolution are likewise proportionately greater.

The Adsorption of Glycine by Aspirin at the Aspirin: Water Interface

Outline of Technique

If an aqueous solution of glycine, saturated with aspirin, is allowed to flow through a column of aspirin crystals the effluent at first contains no glycine but, as more solution is passed through the column, the glycine content rises until eventually the effluent composition is the same as that of the solution entering the column. The lag in arrival of the glycine is partly due to the displacement of the water originally in the column by the glycine solution and partly to the adsorption of glycine by the aspirin crystals. The variation of glycine concentration of the effluent with volume is shown for a typical experiment in Figure 1. Point A corresponds to the volume of liquid initially in the column; the shaded area B represents the amount of glycine removed from solution by adsorption on the aspirin; in the region of C the glycine content approaches that of the inflowing solution, shown in Figure 1 by the horizontal line at 0·5 per cent glycine.

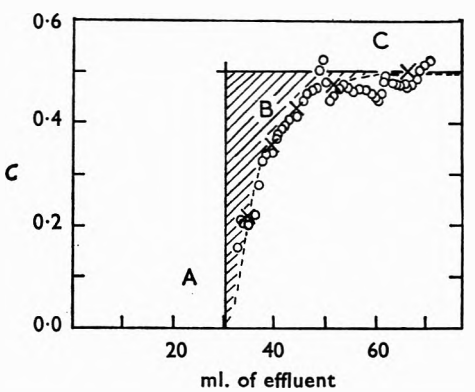

Fig. 1. The progressive change in glycine concentration, C in per cent weight by volume, of the effluent in a typical chromatogram. ○ Experimentally determined values of glycine concentration. –x– Calculated chromatogram profile.

If the shape of curve AC is analysed mathematically a relation for the adsorption isotherm can be obtained. If the size and surface area of the crystals is known this relation can be roughly expressed in terms of the number of layers of glycine molecules at the aspirin: water interface. The data from a number of experiments analysed in this manner are shown in Figure 2. It can be seen that even if the true area of the crystals is ten times the estimated area, which is calculated from the geometry

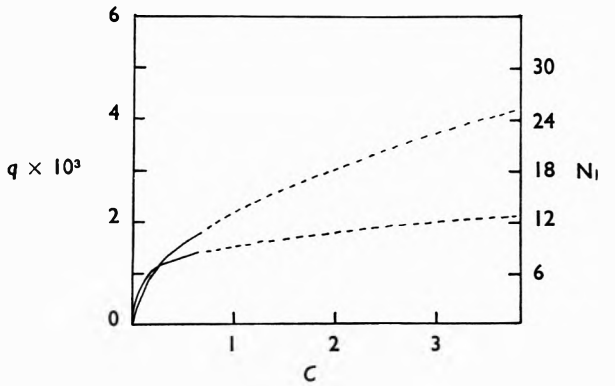

FIG. 2. Adsorption isotherm obtained by analysis of chromatogram profiles. Where q is the amount adsorbed in g./g. of aspirin, and C is the glycine concentration in per cent weight by volume.

—— Portion of isotherm covered by the glycine concentration used.
–·–· Extrapolated portion of isotherm obtained by useof the equation $q = aC^p$ to which portion the isotherm at lower concentrations appears to conform.

Calculation of the number, N_1, of layers of molecules on the aspirin crystal surface is based on the mean geometric and the calculated cross sectional areas of, respectively, the crystals and the glycine molecule.

of the particles, then layers of up to 50 molecules could be obtained under the conditions expected when an aspirin : glycine tablet dissolves in the mouth. It is believed that a layer of even 10 molecules would have a physiological effect inhibiting adhesion of aspirin crystals to the mucosa of the digestive tract. The results are consistent with the observed fact that little taste of aspirin is detected when an aspirin : glycine tablet is held in the mouth before swallowing, in comparison with pronounced acid taste of the ordinary aspirin tablet made from crystals of the same particle size.

To simulate the actual conditions these adsorption measurements have been made on aspirin crystals of particle size similar to that used for preparation of the compound tablets, whereas the use of much smaller particles is to be preferred in adsorption studies on account of the larger surface area. It seems significant to find that the adsorption is measurable under these conditions.

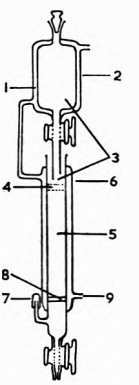

FIG. 3. Chromatography apparatus showing thermostatically controlled, jacketed, adsorption column.

1, Jacket. 2, Reservoir and constant head device. 3, Glycine solution saturated with aspirin. 4, Cotton wool. 5, Aspirin crystals. 6, Constant head. 7, Rubber cap. 8, Sintered disc. 9, Water inlet.

EXPERIMENTAL

The column (Fig. 3) was prepared by packing the aspirin crystals into a glass tube (diameter 1·45 cm., length 70 cm.) into which a sintered

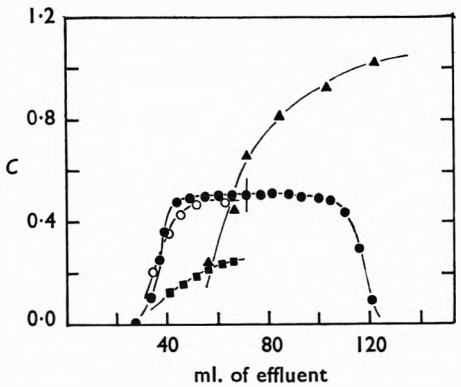

FIG. 4. Glycine chromatograms. Where C_o and C are, respectively, the inflowing and outflowing concentrations in per cent weight by volume.

■ $C_o = 0{\cdot}25$ per cent glycine; 22°, pH 3·0 (Small column).
○ $C_o = 0{\cdot}5$ per cent glycine; 22°, pH 3·0 (Small column).
▲ $C_o = 1{\cdot}0$ per cent glycine; 25°, pH 3·0 (Large column).
● $C_o = 0{\cdot}5$ per cent glycine; 35°, pH 2·0 (Small column).
Start of the elution with glycine free solvent.

glass disc of No. 2 porosity had been fused. A small pad of cotton wool at the top of the column prevented disturbance of the crystals by the inflowing solution. The stoppered reservoir, placed so that the outlet was just below the surface of the liquid in the column, acted as a constant head device, the flow rate being controlled by a tap at the bottom of the apparatus. Both reservoir and column were surrounded by a thermostatically controlled flow of water through a jacket shown in the Figure.

After introducing a weighed amount of aspirin crystals into the column, the latter was gently tapped and saturated aspirin solution was slowly drawn up to displace the whole of the air between the crystals. Saturated aspirin solution was then run in from the reservoir until steady flow conditions were established. The reservoir was then replaced by one containing the appropriate glycine solution saturated with aspirin.

Weighed quantities of effluent were taken at suitable intervals during each run, sampling more frequently when the glycine concentration was changing more rapidly. The samples were assayed for glycine by the ninhydrin method, using a spectrophotometer at a wavelength of 575 mμ.

Measurements were made with solutions containing 0·25, 0·5 and 1·0 per cent glycine at a pH of 3·0 and temperatures of 22° to 35° and with a

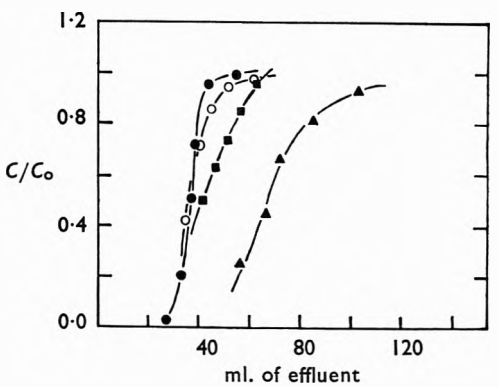

FIG. 5. Reduced glycine chromatograms. Where C_o and C are, respectively, the inflowing and outflowing glycine concentrations in per cent weight by volume.

■ $C_o = 0{\cdot}25$ per cent glycine; 22°; pH 3·0.
○ $C_o = 0{\cdot}5$ per cent glycine; 22°; pH 3·0.
● $C_o = 0{\cdot}5$ per cent glycine; 35°; pH 2·0.
▲ $C_o = 1{\cdot}0$ per cent glycine; 22°; pH 3·0.

0·5 per cent glycine solution at pH 2·0 and 35°. The experimental results are shown in Figures 4, 5 and 6. In the reduced chromatograms of Figure 5 the glycine concentration of the effluent is expressed as the ratio C/C_o where C_o is the concentration entering the column. In order to determine whether the glycine was irreversibly held by the aspirin, columns on to which glycine was adsorbed were washed (eluted) with a saturated solution of aspirin only and the effluent analysed for glycine. From the results of such an experiment illustrated in Figure 4, it would appear that the glycine is easily removed, suggesting that the binding is physical rather than chemical.

The essential points of a mathematical analysis of these measurements are now given in the following paragraphs.

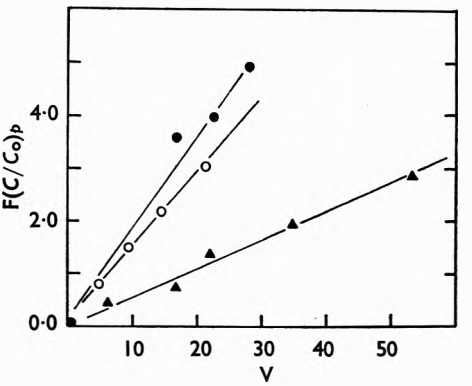

FIG. 6. Test of Gluekauf equation for adequate representation of chromatogram front profile. Values of the function $F\left(\frac{C}{C_o}\right)_p = \mathrm{Log}_{10}\left(1-\left(\frac{C}{C_o}\right)^{1-p}\right)$ for $p = 0{\cdot}25$ are plotted against the effluent volume V, which is in ml.

○ $C_o = 0{\cdot}5$ per cent glycine; 22°, pH 3·0.
● $C_o = 0{\cdot}5$ per cent glycine; 35°, pH 2·0.
▲ $C_o = 1{\cdot}0$ per cent glycine; 22°, pH 3·0.

Gluekauf[5] has deduced an equation relating the concentration to the volume of effluent for solutes having concave adsorption isotherms of the form

$$q = a\,C^p \qquad (1)$$

where q = amount adsorbed,
C = concentration of solute in solution,
p and a are constants depending on the solute, solvent and adsorbent.

The Gluekauf equation is

$$(V-V_t) = -\frac{F}{K(1-p)}\log_e\left\{1-\left(\frac{C}{C_o}\right)^{(1-p)}\right\} \qquad (2)$$

where V = volume of effluent,
V_t = threshold volume (breakthrough volume),
C_o = concentration of solute entering the column,
C = concentration of solute leaving the column at volume of effluent V,
F = flow rate,

and K is a constant, such that 1/K is the time taken for 1/eth of the equilibrium amount of solute to adsorb on the adsorbent.

If this equation holds for a chromatogram, then a plot of $\log_e\left(1-\left(\frac{C}{C_o}\right)^{(1-p)}\right)$ against V will give a straight line whose slope m is given by:—

$$m = -\frac{K(1-p)}{F} \quad .. \quad .. \quad .. \quad (3)$$

Calculations are made with various values for p and the value of p for the best fit is taken.

Figure 6 shows the results of such calculations for a number of chromatograms and it appears that a value of 0·25 (approx.) for p is most reasonable.

The points marked on the curve of Figure 1 have been obtained from equation (2) into which values for p and K have been inserted. It is possible to calculate K from equation (3) since the flow rate for any experiment is known and the slopes of the lines in Figure 6 are measurable.

The value for p of 0·25 has been used to calculate the isotherm shown in Figure 2. For interest the isotherm for $p = 0{\cdot}5$ is also given, since the most probable value of p is between 0·25 and 0·5. The equation to the isotherm is obtained by insertion of the value for p in equation (1). The constant a of the latter equation is then evaluated from any pair of experimental values for q and C.

Equation (1) is the well-known Freundlich adsorption isotherm. This isotherm has been used to correlate the results, with apparent success, even though it may not be strictly applicable to the adsorption phenomena studied. For this reason the extrapolated portions of the isotherms of Figure 2 are shown as broken lines; whilst the continuous line indicates the portion covered by the experimental conditions. This does not, however, detract from the fact that adsorption is taking place as evidenced by the time lag in arrival of glycine in the effluent.

Conclusions

It was suggested that the physiological differences in the behaviour of aspirin and aspirin with glycine should be capable of more precise explanation in physico-chemical terms.

It was also suggested that the basis of two possible and readily tested explanations were concerned with solubility, rate of dissolution and adsorption.

The hypotheses have been put to the test and the results show that aspirin is 15 to 20 per cent more soluble, and that it dissolves more rapidly, in the presence of glycine. Consequently it is to be expected that aspirin in the presence of the latter will be more rapidly transported from the mouth by salivation and swallowing.

The adsorption of glycine on aspirin particles has also been demonstrated. The mechanism of such adsorption is probably closely related to that of the adhesion of aspirin to the mucosal membranes. Consequently the non-adhesion of a glycine coated aspirin particle can be, quite reasonably, anticipated.

The conclusion that marked interaction between glycine and aspirin molecules occurs in solution is fully substantiated by the observed solubility and adsorption phenomena.

Edwards[4] suggests that diffusion is an important factor in determining the dissolution rate. The diffusion process would, therefore, seem to be affected by the interaction of glycine with aspirin, consequently such interactions may have an important influence on the transport and activity of aspirin in the body. Their exact nature and significance must, however, be the subject of further investigation.

Acknowledgements. The authors acknowledge with thanks the useful discussions they have had with Dr. J. Farquharson, Dr. L. J. Edwards and Mr. D. N. Gore; and the assistance they have received from their colleague Mr. D. F. Lawson.

References

1. *Brit. med. J.*, 1955, **2**, 31.
2. Douthwaite, *Lancet*, 1954, **2**, 917.
3. Vining and Kersley, *Brit. med. J.*, 1957, **1**, 444.
4. Edwards, *Trans. Faraday Soc.*, 1951, **47**, 1191.
5. Gluekauf, *J. chem. Soc.*, 1947, 1302, 1315.

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

The adsorption theory best explains the observed facts. Glycine is adsorbed on aspirin during manufacture of the tablets. There was no information on the particle size of the aspirin used in the final product. The proportion of glycine to aspirin was the lowest which would give the required effects. The preparation is subject to hydrolysis. Replacing glycine with sucrose does not disguise the taste of aspirin. The pH of the aspirin-glycine preparation is about pH 2. The glycine had no apparent effect on the adsorption peak of the aspirin.

THE FLASK COMBUSTION TECHNIQUE IN PHARMACEUTICAL ANALYSIS: IODINE-CONTAINING SUBSTANCES

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The flask method for combustion of organic samples has been applied to a number of iodine-containing substances of pharmaceutical interest including the non-staining iodine ointments. The method is both rapid and simple and the accuracy and precision of the results obtained show that it is suitable for routine control purposes and possibly for consideration as an official assay procedure.

The flask combustion technique has aroused considerable interest since the application of the principle was revived by Schöniger in 1955[1]. The method has been used for the determination of many types of substances[2] but to date there has been no attempt to apply it to materials of pharmaceutical interest. The iodine-containing substances of the British Pharmacopoeia and British Pharmaceutical Codex form a group of materials to which the method might be applied with advantage. The flask combustion method described below is simpler and more rapid than the present official methods and an assessment of its applicability, precision and accuracy therefore seemed desirable.

Experimental

The method recommended is applicable to all the substances examined and is based on that described by Schöniger[1,3].

Apparatus. The apparatus consists of a stout-walled 500-ml. iodine flask of resistant glass. Into the stopper is fused one end of a length of platinum wire about 13 cm. long and 1 mm. in diameter. To the free end is attached a piece of 36 mesh platinum gauze measuring $1\frac{1}{2} \times 2$ cm. (Fig. 1).

Reagents. These should be of analytical reagent grade wherever possible. *Bromine solution.* Dissolve 100 g. of potassium acetate in glacial acetic acid, add 4 ml. of bromine and dilute to 1 litre with glacial acetic acid. *Formic acid. Potassium iodide. Sodium hydroxide* N. *Sodium thiosulphate* 0·02 N and 0·01 N. Prepared when required from 0·1 N sodium thiosulphate which has been standardised against potassium iodate. *Starch mucilage* of the B.P. appendix II B.

Method. Accurately weigh a suitable quantity of the sample by difference and transfer to a strip of filter paper (Whatman No. 1 is suitable) which has been folded into three along its length. A convenient size has been found to be 3×5 cm. but this is not critical, provided that both sample and paper burn satisfactorily during the subsequent combustion stage. Enclose the sample in the filter paper by folding in the outer thirds and rolling up the strip. Grip the small packet so obtained in the platinum gauze and insert a narrow strip of filter paper into the roll to act as a fuse.

Moisten the ground glass joint of the flask with water and fill the flask with oxygen after adding 10 ml. of water and 2 ml. of N sodium hydroxide. Ignite the fuse and immediately insert the stopper into the flask. Since a small positive pressure is formed during combustion, hold the stopper firmly in place. Once the sample is burning vigorously turn the flask on its side so as to prevent incompletely burned material from falling out of the gauze into the liquid. As soon as the combustion is complete shake the flask vigorously for about 5 minutes; then place a few ml. of water in the collar of the flask and withdraw the stopper, when the slight negative pressure will suck the water in to wash down the neck. Rinse the stopper, platinum wire, gauze and walls of the flask with distilled water. Add an excess (5–10 ml.) of bromine solution and allow to stand for 2 minutes. Remove the excess bromine by addition of formic acid (about 0·5–1·0 ml.), wash down the sides of the flask with water and sweep out any bromine vapours above the liquid with a current of air. Add 1 g. of potassium iodide and titrate with 0·02 (or 0·01) N sodium thiosulphate, using starch mucilage as indicator.

FIG. 1. Stopper with platinum wire and gauze attached as used in the flask combustion method.

With ointments, the sample is weighed on to a small square of greaseproof paper which is folded so as to completely enclose the material and is then itself folded in filter paper as usual. In some instances a small amount of carbon may deposit on the wall of the flask during combustion but this does not appear to affect the result.

Schöniger[1] suggested a technique whereby the method might be applied to the assay of liquid samples. A glass capillary was used to hold the sample and this was wrapped in paper and then broken immediately before ignition. Some workers[4,5] have made use of gelatin capsules to hold the liquid but in our experience methylcellulose capsules are preferable, since these burn without "spitting" and do not give rise to nitrogenous combustion products. The liquid sample is weighed into one end of the capsule, a piece of rolled filter paper is added to act as an absorbent, the capsule is closed, wrapped in filter paper and ignited as usual.

RESULTS AND DISCUSSION

The method was first tried on *o*-iodobenzoic acid of micro-analytical reagent grade (theoretical iodine content 51·17 per cent) and was shown to give satisfactory recoveries (Table I).

Table I also lists the results obtained on a number of iodine-containing substances used in pharmacy. It will be seen that the mean values obtained by the Flask method and results by the official method (which in each case is that described in the appropriate B.P. or B.P.C. monograph) are in good agreement. Results obtained by two operators showed that the range obtained using the flask method was in most assays

somewhat wider than that using the reference method. To make a more thorough assessment of the variation to be expected when using the flask method three official substances of different type were chosen for special study. The results obtained are listed in Table II. In our

TABLE I

RESULTS OBTAINED BY THE OFFICIAL METHOD AND THE FLASK METHOD ON VARIOUS IODINE-CONTAINING SAMPLES

Substance	Results by official method	Results by flask method			
		Analyst	No. of determinations	Mean	Range
Iodobenzoic Acid ..	51·17 per cent I (Theory)	1 2	9 5	51·1 51·1	50·9–51·2 50·9–51·3
Chiniofon Sodium B.P.	99·1 99·2	1 2	6 4	99·1 99·0	98·9–99·3 98·7–99·4
Di-iodohydroxyquinoline B.P.	98·1 98·3	1	3	98·3	98·1–98·5
Iodoform B.P.C. ..	99·9 100·3	1 2	3 3	99·9 99·8	99·8–100·2 99·7–100·0
Iodoxyl B.P. (I)* ..	85·8; 85·8 85·7; 85·8	1 2	4 3	85·7 85·6	85·2–85·9 85·5–85·7
Iodoxyl B.P. (II) ..	100·0	1	3	100·1	100·1–100·2
Iopanoic Acid B.P. ..	99·3 99·4	1 2	4 2	99·3 99·2	99·1–99·5 99·1–99·2
Pheniodol B.P. ..	99·1	1 2	4 4	98·9 99·0	98·7–100·1 98·7–100·2
Thyroxine Sodium B.P.†	90·2	1 2	5 3	90·2 90·3	90·1–90·5 90·2–90·4
Propyliodone B.P. ..	100·1 100·0	1 2	4 4	100·0 100·0	99·9–100·0 99·8–100·1
Ethyl Iodophenylundecanoate B.P.C.	100·0 100·0	1	3	100·2	100·1–100·3
Tablets of Diiodohydroxyquinoline B.P. ..	0·195 g./tab.	1 2	3 3	0·196 g./tab. 0·197 g./tab.	0·195–0·196 0·197–0·198
Tablets of Pheniodol B.P.C.	0·479 g./tab.	1	3	0·479 g./tab.	0·478–0·480
Iodised Oil Injection B.P.	39·9 per cent I. 40·2 (by fusion)	1	6	40·3	39·9–40·5
Chiniofon B.P. 1948 ..	29·6 per cent I	1	2	29·6	29·4–29·8

All the above determinations were made using 0·02 N sodium thiosulphate and sample weights varying between 10 and 25 mg. to give a titre of about 20 ml. according to iodine content. Results are expressed as per cent compound unless otherwise stated.

* Old and discoloured sample. † Calculated with reference to original material.

opinion these figures show that the method is suitable for routine control purposes, and might be acceptable as an official assay procedure subject to its satisfactory application in other laboratories.

The results obtained on various batches of non-staining iodine ointments are particularly interesting. Although reproducible results were obtained by the flask combustion method (except in Batch 3) it appeared that there was no relation with the B.P.C. method. In view of this the zinc reduction method (B.P.C. 1949, Supplement 1952) and a modified B.P.C. method were also applied. Results by the three comparison

methods showed considerable differences but results by the flask technique were in close agreement with those obtained by the modified B.P.C. method. The figures obtained by the flask method on Batch 3 showed a considerable spread and, in view of the agreement on other samples, it was concluded that the batch was not homogeneous. After the batch had been reworked good agreement was obtained. Duplicate determinations on non-staining iodine ointments can be made within 40 minutes.

TABLE II

A COMPARISON OF THE PRECISION OF THE FLASK AND OFFICIAL METHODS FOR THREE B.P. SUBSTANCES

Substance and method	No of results	Mean results	Range	Standard deviation
Thyroxine Sodium				
Flask	18	90·3	90·1–90·6	0·146
Official	6	90·2	90·1–90·3	0·077
Pheniodol				
Flask	24	99·0	98·7–99·2	0·161
Official	6	99·1	99·0–99·2	0·100
Propyliodone				
Flask	20	100·0	99·7–100·2	0·151
Official	6	100·1	100·0–100·2	0·089

TABLE III

RESULTS OBTAINED BY THE FLASK AND THREE OTHER METHODS ON NON-STAINING IODINE OINTMENTS

Batch No.	Analyst	Results (per cent Iodine)				
		By Flask combustion (50 mg. sample 0·01 N $Na_2S_2O_3$)	Mean	Zinc reduction (B.P.C. Supp. 1952)	B.P.C.	Modified B.P.C.*
1	1	5·10; 5·10; 5·09; 5·06;	5·09	4·90	4·98	5·07
	2	5·11; 5·09; 5·11; 5·10;	5·10	4·92	5·00	
2	1	5·25; 5·23; 5·24; 5·26;	5·25	5·04	5·15	5·24
				4·98	5·08	
3†	2	5·43; 5·80; 6·21; 5·39;		5·25		5·38; 5·52
3 (resample)		5·52; 5·38; 5·54; 5·36;				
3 (reworked)		5·47; 5·44; 5·49; 5·50;	5·48			5·46
4†	1	5·05; 5·03; 5·00; 5·03;	5·03	4·73	4·72	4·99
				4·75	4·74	4·99

* Sample and sodium carbonate packed in two crucibles as for Chiniofon Sodium B.P.
† Batches 3 and 4 contained methyl salicylate.

When the work was started it was considered possible that, because of the small weight of material used for a determination, slight variations in uniformity of samples might significantly affect the results; in practice this was not found out to be the case. All the assays listed above were carried out on routine control samples which had not been pre-treated in any way. The ability of the method to detect non-uniformity in samples so rapidly may be turned to advantage as was demonstrated with the Iodine Ointment.

References

1. Schöniger, *Mikrochim. Acta*, 1955, 122.
2. Macdonald, *Industr. Chem. Mfr.*, 1959, **35** (1), 33.
3. Schöniger, *Mikrochim. Acta*, 1956, 869.
4 Lysyj and Zarembo, *Analyt. Chem.*, 1958, **30**, 428.
5 Corner, *Analyst*, 1959, **84**, 41.

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

PHYTOCHEMICAL CHANGES INITIATED BY INSECTS

PART I. PRELIMINARY WORK ON LEAVES AND "BEAN GALLS" OF *SALIX FRAGILIS* L.

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Received May 1, 1959

The occurrence of leaf galls and the relation between the host plant and the causative insect are discussed. The leaves, galls, and leaves freed from galls of *Salix fragilis* have been examined by one-way and two-way paper chromatography and a separation of the major constituents achieved on powdered cellulose columns. The galls and leaves freed from galls were compared by band chromatography and the results suggest that one phytochemical change initiated by the sawfly is an accumulation of catechins, leucoanthocyanins and a ninhydrin positive substance.

PESTS of cultivated plants have attracted much work in recent years, but less attention has been given to the biological relation between plant and pest. Gall formation which is initiated by insects and mites presents an opportunity to investigate a relation of this kind, especially the chemical changes which may occur. Gall formation is common among higher plants. The galls arise only from meristematic tissues which have received an irritating stimulus and it is this which initiates abnormal growth[1]. An insect supplies the initial stimulus but the host carries the process forward. The commonest insect galls are produced by the *Cynipidae* (gall wasps) and *Tenthredinidae* (sawflies). Gall formation by gall wasps follows larval emergence while with sawflies the gall is already formed before larval emergence. The adult sawfly seems to supply the necessary stimulus during oviposition and the possibility of the stimulus being a chemical one cannot be ruled out[2].

In the Salicaceae, galls are common and frequently sawflies are responsible. The galls formed on leaves may involve inward rolling of the margin, deformity of the lower surface, or abnormal growth within the leaf blade which shows on both surfaces. This latter type of gall is found on *Salix fragilis* as well as other willow species[3], it is known as the "bean gall" and is caused by *Pontania proxima.*

To study the chemical changes initiated by insects in leaves, *Salix fragilis* was first chosen. Information on the constituents of willow leaf galls is scanty. The colouring matters of various insect galls including those of *Salix sp.* have been studied and the pigments eriophyesin and pontanin have been isolated[4]. These are reputed to be glucosides of purpurogallin but as they were isolated from air dried galls they could be artefacts[5]. A hypothetical scheme[6] for which there appears to be no real biochemical evidence has been put forward for the formation of purpurogallin. A comprehensive investigation of the normal leaf

constituents of commonly occurring British willows has not been made although some work on specific glycosides, especially in barks, has been published[7–13]. The presence of leucoanthocyanins has been recorded in the leaves of two species of *Salix*[11]. The constituents of *S. fragilis* leaves have been reported[15] as gallic acid, catechin and quercetin.

Experimental Methods

Material was collected from trees growing near Leatherhead, Surrey, in September. Three types of samples, leaves, galls, and leaves freed from galls were prepared for chemical analysis by two methods. Half of each type of sample was dried at 90°, then powdered. The other half was treated as follows. The material was chopped fine and macerated in 70 per cent ethanol for 2 weeks. The tissue was then filtered, lightly pressed and washed with small quantities of the solvent. The mixed filtrates were finally adjusted to a standard volume with further solvent. Extracts of leaves and of leaves freed from galls contained 20 leaves per 100 ml. and extracts of galls, 40 galls per 100 ml.

Chromatography of Dried Material

Using the method recommended for leucoanthocyanins[16], but with suitable quantities of reagents, powdered leaf, galls, and leaf freed from gall were separately heated with 2 N hydrochloric acid at 100° for 20 minutes and filtered while hot. The filtrate of each was gently shaken with *n*-amyl alcohol and drops of the alcoholic extract applied to the starting line of a paper chromatogram until a distinct pink to brown spot was produced after drying. The ascending technique was used with Whatman No. 1 paper and a single phase mixture (Forestal solvent)[17], water : acetic acid : hydrochloric acid, 10 : 30 : 3, which was run for 20 hours. Chromatograms were examined under ultra-violet light. Chromatographic comparison was made with pure samples of quercetin, myricetin and caffeic acid, while cornflowers were used as a reference source of pelargonidin and cyanidin.

A separation of the major constituents was tried using a glass tube ($\frac{3}{4}$ in. × 10 in.) packed with dry powdered cellulose. Samples of powdered leaf, gall, and leaf freed from gall 5 g., were separately boiled for 30 minutes with 100 ml. of distilled water, filtered and the extraction repeated with a further 100 ml. of the solvent. The mixed filtrates of each were cooled and treated with 20 per cent w/v lead acetate solution until no further precipitation occurred. The lead complex was filtered off and suspended in 100 ml. of absolute ethanol and the lead precipitated by hydrogen sulphide. The lead sulphide was removed by filtration and the ethanol removed from the filtrate on a boiling water bath, the residue taken up in 2 ml. distilled water and applied to the top of the cellulose column. This was developed with distilled water, without reduced pressure, until the faster moving components reached the bottom of the column. It was then extruded and the bands distinguished by ultra-violet light. Tests were made for the major constituents using suitable reagents[18].

Chromatography of Extracts

Extracts of leaf, gall, and leaf freed from gall were examined by two-way chromatography with Whatman No. 1 paper and the ascending technique. Using a micrometer pipette, 0·08 ml. of extract was applied near one corner of the chromatogram, dried and the alcoholic phase of the mixture, *n*-butanol : acetic acid : water, 4 : 1 : 5 run for 20 hours in one direction. After drying the chromatogram in a current of hot air the second solvent, distilled water, was run for 3 to 4 hours at right angles.

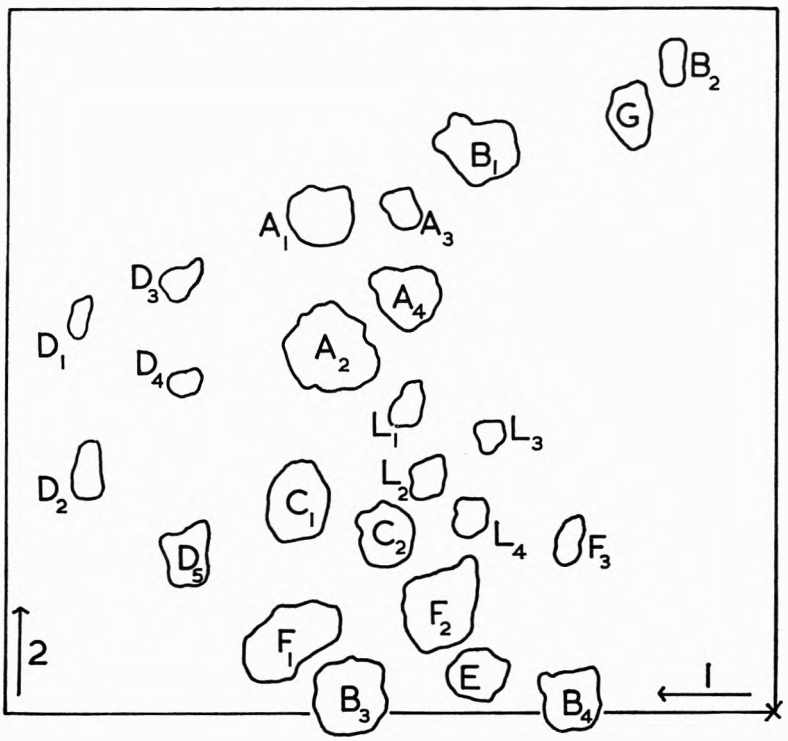

FIG. 1. Two-way chromatogram showing the separation of constituents present in extracts of leaves, galls, and leaves freed from galls. (see Table I for key to spots).

1 = *n*-butanol :acetic acid :water, 4:1:5.

2 = distilled water.

Chromatograms were dried and examined in visible light, then in ultra-violet light before and after exposure of the papers to the vapour of ammonia. Replicate chromatograms were done and spots revealed by spraying the papers with suitable chemical reagents[19,20].

Band chromatography on paper sheets was tried with the object of separating constituents of the extracts of gall, and leaf freed from gall and also to enable semi-quantitative estimations to be made. For both extracts 1 ml. was applied to the length of a starting line measuring 12 in. on a sheet of Whatman 3 MM paper and after drying, a further

TABLE I

EXTRACTS OF LEAVES, GALLS, AND LEAVES FREED FROM GALLS, COLOUR TESTS APPLIED TO TWO-WAY CHROMATOGRAMS

Key to spots in Fig. 1	Visible light	Ultra-violet light	NH_3/Ultra-violet light	*a* $NaNO_2$ then NaOH	*b* D.S.A.	*c* Vanillin	*d* Ferric alum/NH_3	*e* Ferric-ferri-cyanide	Remarks
A_1, A_2 A_3, A_4	None	Blue	Green	Pink	Orange	None	Green to black	Blue	Chlorogenic acids
B_1, B_2 B_3, B_4	None	Violet	Intense violet	None	None	None	None	None	Non-phenolic
C_1, C_2	None	None	None	None	Yellow	Red	Green to blue	Blue	Catechins
D_1, D_2 D_3, D_4 D^5	None	Blue	Blue/green	None	None	None	None	Blue	Minor phenolic constituents
E*	Red	—	—	—	—	—	—	—	Anthocyanin
F_1, F_2	Yellow	Brown	Yellow	Yellow	Orange	None	Brown to yellow	Blue	Flavonoid
F_3	None	None	Yellow	Yellow	None	None	None	None	Glycosides
L_1, L_2 L_3, L_4	None	None	None	None	Yellow	Red	Green	Blue	Leucocyanidins

* Pigment E detectable in gall only. (See footnote on page *228 T.*)

NOTES:—
a = $NaNO_2$ 1 per cent w/v in 10 per cent acetic acid followed by N NaOH.
b = diazotised sulphanilic acid reagent B.P.
c = equal volumes of hydrochloric acid B.P. and a mixture of vanillin 2 per cent w/v + acetyaldehyde 1 per cent v/v in 95 per cent ethanol.
d = ferric alum 0·2 per cent w/v in water, followed by ammonia vapour.
e = ferric chloride 0·3 per cent w/v in water and potassium ferricyanide 0·3 per cent w/v in water, equal volumes freshly mixed.

1 ml. was similarly applied. The solvent, distilled water, was run for 5 to 6 hours, the paper dried and the positions of the bands revealed by ultra-violet light. Each band was cut out, eluted with 10 ml. of cold absolute ethanol and the solvent removed on a boiling water bath. The fractions so obtained were analysed by two-way chromatography to check the components of each band. Replicate chromatograms were sprayed using the vanillin and ninhydrin reagents.

Results

Dried Material

With leaf, gall, and leaf freed from gall one-way chromatograms showed similar results. There were two spots which were bluish under ultra-violet light, one of which coincided with caffeic acid. Two further spots were bright yellow, one of which coincided with quercetin. Another

TABLE II

BAND CHROMATOGRAPHY ON PAPER SHEETS OF EXTRACTS OF GALLS, AND LEAVES FREED FROM GALLS

Bands from top of sheet	Run in distilled water 5 to 6 hr.	Run in alcoholic phase of *n*-butanol/acetic acid/ water, 4 : 1 : 5, 20 hr.
	Band components	
1	B_1, B_2, G	D_1, D_2
2	A_1 to A_4	D_3, D_4, D_5
3	Narrow bands	F_1
4	Minor	A_1, A_2, B_3
5	Constituents	A_3, A_4, F_2
6	D_5 and vanillin positive constituents	B_1
7	F_2	B_4, F_3
8	F_1	G
9	B_3, B_4	B_2

spot, which was red in visible light coincided with cyanidin. The cellulose columns produced a separation of the constituents into three distinct bands. The first band was of constituents which are non-mobile in water and showed a deep violet fluorescence. The second band gave a velvety brown fluorescence and an aqueous extract of it produced a red colour with vanillin reagent, a red colour when boiled with 2 N hydrochloric acid and a crimson colour with the magnesium : hydrochloric acid test. The third and largest band gave a strong blue fluorescence and an aqueous extract of it produced a green colour with ferric ammonium sulphate reagent.

Extracts

With leaf, gall, and leaf freed from gall two-way chromatograms showed similar results except that substances C_1 and C_2, L_1 to L_4 and G (see Fig. 1) were clearly visible only on chromatograms prepared from gall extracts, which alone showed the presence of the red pigment E. The results of colour tests applied to chromatograms are shown in Table I and Figure 1. Additional colour tests were applied to chromatograms and the results obtained were as follows. After spraying with 0·1 per cent

w/v ninhydrin in 10 per cent acetic acid and heating for a few minutes in a current of hot air, the spot G having a violet colour was revealed. This substance has an R_F value of 0·7 (approx.) in phenol saturated with water and does not coincide with either aspartic acid or glutamic acid. Red spots were not produced when chromatograms were sprayed with 2 N sulphuric acid, and then heated in a current of hot air and pink spots were not produced when potassium cyanide 1 per cent w/v in water was used as a spray reagent. Band chromatograms of gall extracts showed wider and more strongly coloured vanillin and ninhydrin positive bands than corresponding chromatograms of extracts prepared from leaf freed from gall. Except for these differences chromatograms were similar and the components of each band are given in Table II. Nine bands were produced but the faster moving bands 1 and 2 were wavy in outline owing to interfering substances. The vanillin positive constituents formed a single band (6) which also contained substance D_5. A different solvent, the alcoholic phase of the mixture *n*-butanol : acetic acid : water, 4 : 1 : 5 can be used as running solvent for 20 hours. This gives uniform separation of the constituents again into 9 bands distinguishable by ultra-violet light. The vanillin positive constituents C_1 and C_2 overlapped bands 3 and 4 and the other vanillin positive constituents overlapped bands 6 to 8 and gave a red colour when boiled with 2 N hydrochloric acid.

Discussion

Dried Material

For leaf, gall, and leaf freed from gall, the results of one-way chromatography and of tests on fractions from cellulose columns indicate that two types of flavonols are present, one of which is based on quercetin. Leucoanthocyanins are present and are probably based on cyanidin and these constituents account in part for the red colour obtained by the vanillin reaction. No further conclusions could be made from this preliminary work and hence the necessity for more detailed chromatographic analysis.

Extracts

From the results of colour tests which were applied to two-way chromatograms and the pattern of spots which were revealed it is suggested that there are no significant qualitative chemical differences between the extracts of leaf, gall, and leaf freed from gall. It is probable that the group of substances A_1 to A_4 are chlorogenic acids; that the pair of vanillin positive constituents C_1 and C_4 are (+)-catechin and (−)-epicatechin respectively and the other group of vanillin positive constituents are leucocyanidins. The substances F_1 and F_2 are flavonoid glycosides but no further conclusions can be made about their identity except that they are based on different aglycones. The red gall pigment E* is possibly an anthocyanin but it should be isolated from fresh galls rather than dried material and separated from leucoanthocyanins to make precise identification possible. The absence of gallic acid and gallocatechins, as

* This pigment has now been shown to be present in young normal leaves.

suggested by the negative potassium cyanide test, does not lend support to Nierenstein's scheme[6] for the chemical relation between the plant and the sawfly, as gallic acid features as an intermediate in this scheme. As the material was obtained from trees in September, failure to detect salicin and saligenin by the sulphuric acid test cannot be taken as proof that these are absent at earlier stages in growth. The results of band chromatography indicate that the method effects a semi-quantitative separation. As the bands of vanillin positive constituents and the ninhydrin positive substance G are wider and more distinct on chromatograms of gall extracts then corresponding chromatograms of extracts prepared from leaf freed from gall, it is assumed that a greater accumulation of catechins, leucoanthocyanins and the ninhydrin positive substance G occurs in the gall than in the leaf.

Acknowledgement.—I wish to acknowledge the advice of Dr. E. A. H. Roberts (Indian Tea Association Research Laboratory) and the receipt from him of three marker substances.

References

1. Swanton, *British Plant Galls*, Methuen, London, 1921.
2. Imms, *Insect Natural History*, 2nd Edn., Collins, London, 1956, p. 170.
3. Carleton, *J. Linn. Soc.* (*Zoo*), 1939, **40**, 575.
4. Nierenstein and Swanton, *Biochem. J.*, 1944, **38**, 373.
5. Paech and Tracey, *Moderne Methoden der Pflanzenanalyse*, Springer-Verlag, Berlin, 1955, **3**, 352.
6. Nierenstein, *The Natural Organic Tannins*, Churchill, London, 1934, p. 127.
7. Rabate, *Bull. Soc. Chim. biol.*, 1935, **17**, 439.
8. Fujikawa and Nakajima, *J. Pharm. Soc. Japan*, 1948, **68**, 175.
9. Bridel and Beguin, *Bull. Soc. Chim. biol.*, 1926, **8**, 901.
10. Wattiez, *ibid.*, 1931, **13**, 658.
11. Sakai, Tpsurumi, Eno and Inukai, *Bull. Inst. Phys. Chem. Res.* (*Tokio*), 1943, **22**, 868.
12. Zemplen, Bognar and Fzekeoy, *Ber.*, 1943, **76B**, 386.
13. Kariyone, *J. Pharm. Soc. Japan*, 1942, **62**, 514.
14. Bate-Smith and Metcalfe, *J. Linn. Soc* (*Bot*), 1957, **55**, 669.
15. Wehmer, *Die Pflanzenstoffe*, 2nd Edn., Jena, 1929, **1**, 200.
16. Bate-Smith, *Biochem. J.*, 1954, **58**, 122.
17. Harbourne, *J. Chromatog.*, 1958, **1**, 473.
18. Paech and Tracey, *Moderne Methoden der Pflanzenanalyse*, Springer-Verlag, Berlin, 1955, **3**, 471.
19. Roberts and Wood, *Biochem. J.*, 1951, **49**, 414.
20. Hillis and Swain, *J. Sci. F. & Agric.*, 1959, **10**, 135.

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

GAS CHROMATOGRAPHY IN ROUTINE PHARMACEUTICAL ANALYSIS

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The application of gas chromatography to the determination of various volatile constituents in a range of pharmaceutical preparations is described. The method is simple, rapid and reliable and has been used to assay camphor, menthol, diethyl phthalate and a number of volatile oils.

In pharmaceutical analysis the direct application of gas chromatography is obvious, particularly for the examination of solvents and isolates from volatile oils, or even for the determination of the main constituents of volatile oils, provided the results can be related to those obtained from the more conventional analyses now standard. There is little reference in the literature, however, to its use in this field—in a bibliography of 653 references up to July 31, 1958[1], barely a dozen of pharmaceutical interest are to be found and these are almost entirely concerned with the examination of volatile oils and flavourings.

Some modification of the recognised technique is necessary to apply it to the determination of the volatile constituents of pharmaceutical compounded preparations, particularly aqueous solutions containing considerable amounts of non-volatile compounds. Further, to make the most efficient use of the technique in the important aspect of time saving, several columns of different lengths and stationary phases maintained at various temperatures should be available for coupling when required to a single recording system. It is sometimes advantageous to have two identical columns operating so that a component with a small retention volume can be determined quickly on one column whilst unwanted components of a sample are being eluted from the other.

The application of the technique to the determination of chloroform in aqueous pharmaceutical preparations has been reported[2], and the equipment with slight modification has since been used for the determination of water in pastes, ointments and creams[3]. The purpose of this paper is to describe the extension of the technique to the determination of various volatile constituents in a wide range of pharmaceutical materials.

Experimental

Equipment

The equipment used is of conventional design, except for modifications necessitated by the fact that the non-volatile materials present in many of the preparations would quickly block the column. As frequent changing of the column cannot be tolerated for control purposes it was necessary to devise and build an injection system to overcome this difficulty. Such a system has already been described[2].

The principle adopted generally is that of using a small removable cap filled with supporting medium and positioned immediately before the chromatographic column. This cap, in which the non-volatile components are trapped, is easily removed when necessary and replaced by a freshly-filled one, the whole operation taking but a minute or so. One form of the device is used with a micropipette method of sample injection and another form utilises a conventional hypodermic syringe injection through a rubber cap. This system of removing non-volatile materials obviates any need for distillation or solvent extraction, and saves time.

The detector used is a thermal conductivity cell with platinum wires 4 in. long and 0·001 in. thick of nominal resistance 25 ohms, the wires in the two channels being matched to within 0·1 ohm. The detector current is 200 mA. and the output is recorded on a Honeywell-Brown or Sunvic recorder with 2·5 mV. full-scale deflection and chart speed of 12 in. per hour.

Calibration

Although many samples contain a number of volatile components we have in general been determining only one of these and to avoid the necessity of calibrating the detector by measuring the peak areas of each component an internal standard procedure has been adopted throughout. With such a procedure it is not necessary to ensure that a standard amount of sample is transferred to the column for each determination and this has proved a useful advantage as many of the samples are thick suspensions or viscous liquids. In all cases peaks are sharp and symmetrical and therefore heights rather than areas can be used for calibration and this is an advantage in routine applications.

Standard solutions, containing known concentrations of the component being determined are prepared and the ratios of the peak height of this material and the internal standard are measured. Calibration curves are then obtained by plotting these ratios against concentration, and working ranges have been chosen to produce linear calibration curves, passing through the origin, except in the particular case of determining water.

Despite rigorous maintenance of operating conditions the calibration curves are subject to slight changes in slope from day to day, but because of their linearity it has only proved necessary in practice to check one or two points before use.

The Determination of Camphor, Menthol and Volatile Oils

The stationary phase used in these determinations is squalane, 2, 6, 10, 15, 19, 23-hexamethyltetracosane, and a column of this material has already been operating continuously at 130° for nine months without any noticeable signs of deterioration.

Standards and samples each containing 2 per cent of ethylbenzene as the internal standard are chromatographed under the following conditions.

Column length—7 ft. Column temperature—130°. Stationary phase —20 per cent of squalane on 100–120 mesh Celite. Sample size—30 μl. Carrier gas—4 : 1 hydrogen/nitrogen mixture flowing at 100 ml./min.

The column characteristics under these conditions are as follows. Height equivalent to a theoretical plate—0·16 cm. Retention volume (ethyl benzene)—270 ml. Retention volume (camphor)—1200 ml. Retention volume (menthol)—675 ml.

The Determination of Diethyl Phthalate

As diethyl phthalate has a high boiling point (296°) it is necessary to carry out the chromatography at a high temperature and the choice of stationary phases is limited but we have found Arylan S90, sodium dodecyl benzene sulphonate, suitable for continuous use at 225°.

Standards and samples each containing 1 per cent of α-phenyl-*n*-propanol as the internal standard are chromatographed under the following conditions. Column length—6 ft. Column temperature—225°. Stationary phase—30 per cent of Arylan S.90 on 36–85 mesh Chromosorb. Sample size—30 μl. Carrier gas—4 : 1 hydrogen/nitrogen mixture flowing at 60 ml./min.

The column characteristics are as follows. Height equivalent to a theoretical plate—0·37 cm. Retention volume (α-phenyl-*n*-propanol)—695 ml. Retention volume (diethyl phthalate)—960 ml.

Results and Discussion

Methods for the determination of camphor in galenicals usually depend on the optical rotation of natural camphor, or on its ketonic nature and have several disadvantages. We have shown that gas chromatography offers a reliable and speedy alternative, an assay taking about 20 minutes. It has been possible to determine camphor in a variety of samples at concentrations greater than 1 per cent, the normal working range being 1 to 10 per cent. The replacement of the thermal conductivity method of detection by an ionisation detector[4,5] should enable as little as 0·1 per cent to be assayed.

Table I shows the results obtained on a variety of samples and the good agreement between these and the chemical results or theoretical concentrations shows the reliability of the gas chromatographic procedure. The chromatographic results are the mean of duplicate determinations, the average divergence from the mean being $\pm$ 1·5 per cent.

Menthol, like camphor, can only be determined at present at concentrations greater than 1 per cent and the use of the technique is therefore limited. In preparations where the concentration of menthol is less than 1 per cent it has proved possible to identify it but not to determine it quantitatively but here again the introduction of the ionisation detector should overcome this difficulty. Liniment of Methyl Salicylate and Eucalyptus B.P.C. and Compound Ointment of Methyl Salicylate B.P.C. contain 5 per cent and 10 per cent of menthol respectively, but the methyl salicylate has a similar retention volume under the conditions employed and completely masks the peak due to the menthol. We have

determined menthol in Oil of Peppermint, and our results agree very well with those obtained by the official B.P. method. Also the amount of oil of peppermint in a number of flavouring compounds, such as one containing equal amounts of oil of peppermint and oil of aniseed has been determined. In these cases the menthol peak was used for measurement but because of the variable amount of menthol in oil of peppermint it was necessary to use as standard the actual batch of oil used in manufacture.

In simple formulations such as Spirits of Aniseed we have determined the volatile oil content fairly accurately using as standard the batch of oil used in the preparation. The need to use the actual manufacturing batch of oil is a disadvantage in applying this technique but is unavoidable

TABLE I

COMPARISON OF CHEMICAL AND GAS CHROMATOGRAPHIC RESULTS AND THEORETICAL VALUES

Sample	Theory	Camphor per cent	
		By gas chromatography	Chemical
Camphor Water Conc. (*a*)	4·0	3·85	3·91
(*b*)		4·13	
Chloral with Camphor	57·1	57·6	59·0
Cold Sore Remedy (*a*)	3·0	2·88	
(*b*)		3·05	
(*c*)		3·02	
Compound Ointment of Capsicum	10·0	10·3	9·8
Hard Ointment of Camphor	6·0	5·95	6·05
Liniment of Belladonna Meth.	5·0	4·3	6·5
Liniment Camphor	20·0	21·2	20·6
Liniment of Camphor Ammon.	12·5	13·0	12·3
Liniment Soap	4·0	3·9	
Liniment Turpentine Acetic	8·2	8·0	
Nasal Drops Chlorbutol	1·37	1·2	
Nasal Drops Chlorbutol and Menthol	1·37	1·3	
Solution Camphor and Oil Aniseed	5·0	4·86	4·95
Spirits of Camphor	10·0	9·98	9·72

because of the variable nature of volatile oils. We have prepared accurately, samples of Spirits of Aniseed, Cinnamon, Juniper, Nutmeg and Rosemary, each with 10 per cent of volatile oil and examined them under the same conditions as for camphor. The average divergence from the theoretical concentration was 1 per cent with a maximum divergence of 3 per cent.

In many toilet waters and allied preparations where the perfume essential oils or the perfume synthetic chemicals or both amount to less than 5 per cent, diethyl phthalate is commonly added as a denaturant, usually at a concentration of 1 per cent v/v. The chemical determination of this compound is a lengthy procedure but it can be determined by gas chromatography in about 20 minutes.

We have examined a number of samples, including lavender water, eau de Cologne, and an oily brilliantine, with theoretical concentrations of 1·00 per cent to 1·02 per cent and the concentration found has varied from 0·99 per cent to 1·05 per cent. To obtain an idea of the reproducibility of the method one particular sample was examined in triplicate and results of 0·99, 1·02, 0·98 per cent were obtained, for which the mean is 1·00 per cent, and the coefficient of variation is 2 per cent. A 1 per cent standard solution when examined on 4 separate days gave results of

0·69, 0·68, 0·67, 0·68 for the ratio of the peak heights of the diethyl phthalate to the internal standard, α-phenyl *n*-propanol.

It has also proved possible to determine diethyl phthalate in solidified perfume sticks. The procedure adopted was to melt sufficient to produce about 10 ml. of liquid and transfer this to a 10 ml. graduated flask containing 0·1 ml. of internal standard, the flask being slightly warmed so as to keep the sample in the liquid state. The micropipette injection system was used and the pipette was again slightly warmed. In this way the sample itself plus the internal standard was transferred directly to the column and no extraction of the diethyl phthalate was required. For two sticks containing theoretical amounts of 0·99 per cent and 1·07 per cent respectively the results obtained were 1·0 per cent and 1·07 per cent.

References

1. *The Chromatography of Gases and Vapours.* A Bibliography. The British Petroleum Co. Ltd., Research Station, Sunbury-on-Thames.
2. Brealey, Elvidge and Proctor, *Analyst*, 1959, **84**, 221.
3. Elvidge and Proctor, *Analyst*, 1959. In the press.
4. Lovelock, *J. Chromatography*, 1958, **1**, 35.
5. McWilliam and Dewar, in *Gas Chromatography*, edit. Desty, Butterworth's Scientific Publications, London, 1958, p. 142.

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

Amplification of the output of the thermal conductivity detector was suggested as a better means of increasing sensitivity than the use of an ionisation detector, which was considered too sensitive, although the latter had the advantage in not being so sensitive to fluctuations in gas flow rate. Background interference could originate from columns that had been used for some time.

A NOTE ON THE USE OF MEMBRANE FILTERS IN STERILITY TESTING

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Filtration techniques have been applied advantageously in testing antibiotics and certain other medicaments, including oily solutions, for sterility, on the one hand by eliminating any residual bacteriostatic activity and on the other by eliminating interference through precipitation or the development of cloudiness. Membrane filters have been shown to be satisfactory for this purpose, and in some respects they are superior to asbestos pad filters.

Since their introduction a few years ago in the bacteriological examination of water supplies[1,2], membrane filters have become established tools for assessing small numbers of micro-organisms. They have been used for counting bacteria in the air[3], for counting surviving bacteria in the air[3], for counting surviving bacteria in certain disinfection tests[4] and have also been advocated in the sterility testing of antibiotics[5]. This communication records some of our experiences and findings in the testing of antibiotics and of oily solutions over about the last two years. Suitable membranes are made in this country by Courtaulds Ltd. and marketed by Oxo Ltd.; they are of such a porosity as will retain all, or very nearly all, of the bacteria present in a given fluid sample.

The technique of membrane filtration consists simply of filtering a known volume of the sample to be examined through a single membrane, suitably mounted in a modified Seitz-type filter holder (Gallenkamp), and then culturing the membrane either on the surface of a prepared nutrient agar plate or by immersion in a liquid nutrient medium. In surface culture, a quantitative assessment can be made, usually by a direct colony count after incubation (it can also be done microscopically after staining[6]), but with liquid media the result is qualitative only; this, however, is normally adequate for sterility testing purposes.

Experimental

The organisms used in the experimental work here reported were a Gram-positive spore former of the *Bacillus subtilis* type, *Staphylococcus aureus* (F.D.A. disinfectant testing strain), and *Chromobacterium prodigiosum* (*Serratia marcescens*).

Efficacy of Membrane Filters

Preliminary tests with membrane filters showed that they could not always be relied upon to retain all of the organisms in a given sample. For this reason, membrane filters were discarded for several years in favour of asbestos pad filters, but more recent tests showed that membranes would allow organisms to pass through only when the inoculum

was numerically high; with small inocula of between 10 and 500 viable cells (the level of contamination most likely to be found in sterility testing) the filtrate was always sterile. Experiments on the recoveries of different test organisms on the membranes themselves confirmed this, and typical recoveries of *Chr. prodigiosum* are given in Table I. These

TABLE I

RECOVERY OF *Chr. prodigiosum* BY MEMBRANE FILTRATION

Numbers of organisms in 100 ml. sample		
Estimated inoculum	Colonies recovered on membrance	Growth in filtrate
9	7	Nil
10	11	"
11	8	"
17	18	"
44	42	"
50	48	"
70	75	"

results were obtained by filtering 100 ml. amounts of the dilute suspensions into sterile bottles each containing 100 ml. of nutrient broth, washing twice with 100 ml. of sterile water or saline, disconnecting each filter from its bottle and then incubating, at 37°, the filtrates for 7 days and the membranes by the agar surface culture method for 2–3 days.

Removal of Antibiotics by Washing

One advantage of the filtration technique is that it allows residual inhibitory substances to be effectively removed before the final culturing, and this applies particularly to the antibiotics other than penicillin. To

TABLE II

REMOVAL OF NEOMYCIN FROM FILTERS BY WASHING

Sample	Neomycin in washings (u./ml.) from			
	Membrane filter		Asbestos pad filter	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Original solution ..	c̄13,000	c̄13,000	c̄13,000	c̄13,000
1st 100 ml. wash ..	140	107	430	360
2nd " " " ..	12	<10	17	15
3rd " " " ..	<10	<10	<10	<10

illustrate this, 100 ml. amounts of a 2 per cent solution of Neomycin Sulphate (i.e., containing about 13,000 u./ml.) were filtered through membrane or asbestos pad filters. The filters were then washed with three portions of 100 ml. of sterile saline and the neomycin content of each of the washings assayed by the standard biological method. The results are given in Table II; they illustrate the superiority of the membrane filter in this respect.

Recovery of Organisms from Antibiotic Solutions

One hundred ml. amounts of solutions of Streptomycin Sulphate (20 per cent) and of Neomycin Sulphate (2 per cent) were inoculated with

small numbers of spores of *B. subtilis* and filtered through separate membranes. Each membrane was washed with two 100 ml. amounts of sterile saline and then either incubated on an agar plate surface or cut in two and each portion incubated in 250 ml. of nutrient broth. The results, summarised in Table III, show a high recovery of organisms from the streptomycin solution, particularly in the qualitative tests, when an estimated inoculum even as low as 5 cells gave a positive response. The results with neomycin were not as encouraging, but these were presumably due to the lethal action of the neomycin, because when the liquid media showing negative responses were inoculated lightly at the end of the incubation period there was an immediate vigorous growth.

TABLE III

RECOVERY OF ORGANISMS FROM ANTIBIOTIC SOLUTIONS

Antibiotic solution (per cent)	Estimated inoculum in 100 ml.	Colonies recovered on filter	Growths in 250 ml. broths
Streptomycin Sulphate 20	9	4	
	25	21	
	28	20	
	5		++
	25		++
	28		++
	33		++
Neomycin Sulphate 2 ..	18	3	
	28	0	
	7		++
	9		++
	28		−−
	28		+−

Test organism—spore-forming Gram-positive bacillus.

Moreover, in one test with the agar plate method, there was a recovery of 60 colonies from an estimated inoculum of 67 spores when the test spores were inoculated into the last washing water.

The absence of bacteriostatic carry-over was further confirmed in a series of experiments in which, after filtering different lots of the antibiotic solutions and washing as before, the membranes were transferred to 250 ml. of culture medium, which was then inoculated lightly (5 to 180 viable cells) with either *Staph. aureus* or *Chr. prodigiosum*. In each of 13 experiments, there was no apparent inhibition of growth.

Recovery of Organisms from Oils

Fifty ml. amounts of arachis oil and of stilboestrol solution in oil were inoculated each with one small loopful of a dilute aqueous suspension of *B. subtilis* spores, and shaken to disperse the organisms. Each of the 50 ml. amounts was then filtered through a dry membrane and the membrane incubated in the usual way. Ten such experiments were made, and with inocula even as low as 8 to 10 spores there was a positive response in every case, growths occurring in periods ranging from 24 hours to 4 days.

In further experiments, the introduction of an aqueous phase was avoided by using a small inoculum (10 to 100 spores) of a freeze-dried

culture of *B. subtilis*. The culture was first suspended in oil and the appropriate amount added to samples of arachis oil and oily solutions of progesterone and stilboestrol; these were then filtered through membranes as before. In each case the test organism was recovered, and the filtrate was sterile.

Discussion

The principal advantage of the membrane filtration method in sterility testing is that having filtered the sample, the membrane can be washed free from any inhibitory or other interfering substances in the original solution; thus the amount of culture medium normally required to dilute out any bacteriostatic effect in the medicament can be much reduced. It is, therefore, particularly useful in testing the antibiotics such as streptomycin and neomycin, for which there is no known inactivator. The method also allows the strongly adsorbed bacteriostats such as the organic mercurials and the quaternary ammonium compounds to be "inactivated" on the filter by washing with a suitable inactivating solution rather than by the more cumbrous and less desirable method of adding the inactivator to the final culture medium. In this respect, membranes are superior to asbestos pad filters because the latter, being fibrous and relatively thick, absorb and retain sufficient of the active material to exert some bacteriostatic action in the final test; moreover the filtration time is rather lengthy.

A further advantage of the method is that it often eliminates the necessity for subsequent subculturing at the end of the normal incubation period, and thus economises in testing time, when the preparation under test produces a cloudiness or precipitate. It can, therefore, be applied to the testing of drugs such as oils and oily solutions (but not oily suspensions), thiopentone sodium and certain of the insulin preparations, all of which cause some cloudiness and precipitation in the medium and so mask any bacterial growth which may have taken place.

The testing of oils for sterility by the normal methods has never been considered satisfactory, largely because of the difficulty of recovering contaminating organisms present in the non-aqueous phase. Here again membrane filters have proved useful, largely because they allow nearly all of the oil to be filtered away and so permit any organisms present more easily to enter the aqueous phase and develop normally. It also helps in subsequent cultural manipulations by eliminating any tendency towards emulsification and the development of opalescence, thus avoiding the need for subsequent subculturing.

A disadvantage of the method is that it requires skill in aseptic handling, otherwise any contaminating organism gaining access during the filtration or subsequent transfer of the membrane to its culture medium will show itself as a false-positive. For this reason, a sterile room is needed with a suitable aseptic screen, or better still the sealed screen technique should be employed[7]. Low recoveries have also been reported in some instances with phenol-damaged organisms[8].

REFERENCES

1. Goetz and Tsuneishi, *J. Amer. Water Wks. Ass.*, 1951, **43**, 373.
2. Taylor, Burman and Oliver, *J. appl. Chem.*, 1953, **3**, 233.
3. Gohar and Eissa, *Z. Hyg. InfektKr.*, 1957, **143**, 364.
4. Hess, Ph.D. Thesis, University of Basel, 1958.
5. Holdowsky, *Antibiot. and Chemother.*, 1957, **7**, 49.
6. Ehrlich, *J. Bact.*, 1955, **70**, 265.
7. Royce and Sykes, *J. Pharm. Pharmacol.*, 1955, **7**, 1046.
8. Harris and Richards, *J. appl. Bact.*, 1958, **21**, 86.

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

The membranes were unsatisfactory with suspensions but had been used regularly for other injectable preparations. Volumes as small as 1 ml. could be used for sterility testing. Recovery had only been studied with a small inoculation of anaerobic organisms. It was not possible to obtain a uniform dispersion in oil of freeze-dried oil suspensions of organisms. Organisms recovered from oils could not be grown on the filters, and suspension of the membrane in a culture medium was necessary. The viscosity of solutions had little influence on the time of filtration, and membranes were much faster than Seitz pads as filters. The amount of any antibacterial agent that might be concentrated in the membrane after filtration of the small quantities of aqueous solutions used, should not be sufficient to influence the result. The membrane method had been used for testing for sterility ox and horse sera in quantities up to 50 ml., but it cannot be used for preparations containing waxes, because of the effects of the organic solvents on the organisms.

THE PREPARATION AND EVALUATION OF SOME PHENOLIC ETHERS AS ANTIFUNGAL AGENTS

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A series of phenolic ethers, derivatives of hydroxybenzoic acids, benzaldehydes and acylophenones have been prepared and tested *in vitro* for antifungal action. The highest activity was found in those compounds having an amyloxy group *ortho* to a carbonyl radical. Data are presented on the *in vivo* antifungal activity and toxicity of 2-*n*-amyloxybenzamide and 2-*n*-amyloxyacetophenone.

Routine screening of compounds for fungistatic activity had previously shown 2-*n*-amyloxybenzamide to be the most active of a series of salicylamide derivatives[1]. This work has been extended to cover corresponding *meta* and *para* alkoxybenzamides which have been shown to be considerably less active than the *ortho* derivatives described in our earlier paper. In view of the high *in vitro* fungistatic activity of the 2-alkoxybenzamides, a series of related 2-alkoxy compounds, in which the amide group was replaced by other carbonyl radicals, was prepared and many of these also showed pronounced fungistatic properties. 2-*n*-Amyloxyacetophenone was the most promising of the compounds examined, and together with 2-*n*-amyloxybenzamide has been subjected to more detailed biological examination.

Experimental

Chemical

The new compounds are listed in Table I.

Unless otherwise stated, the alkyl ethers were prepared from the appropriate phenol by the following general method. The phenol (1 mole) was reacted with an alkyl bromide (1 mole) in boiling ethanolic sodium ethoxide (1 mole). After the mixture had been refluxed for up to 24 hours, the ethanol was removed by distillation to give a residue which was washed with 2N sodium hydroxide. The alkyl ether was collected by filtration or ether extraction and purified by either re-crystallisation or distillation. In the alkylation of methyl hydroxybenzoates, methanol was used as solvent in this reaction, in order to eliminate the possibility of ester interchange.

Alkoxybenzoic acids. Methyl alkoxybenzoates were hydrolysed with 10 per cent ethanolic potassium hydroxide to give alkoxybenzoic acids. 2-*n*-Amylmercaptobenzoic acid was similarly prepared from phenyl 2-*n*-amylmercaptobenzoate.

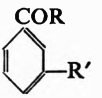
3-*Alkoxy and* 4-*alkoxybenzamides.* The appropriate alkoxybenzoic acid was treated with thionyl chloride (2 equivalents) to form the acid chloride. A solution of the crude acid chloride in benzene was poured into an excess of aqueous ammonia solution and the amide was collected by filtration.

2-*n*-*Amylmercaptobenzamide* was prepared in the same way from 2-*n*-amylmercaptobenzoic acid.

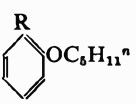
2-n-*Amyloxybenzhydroxamic acid.* Methyl 2-*n*-amyloxybenzoate (22·2 g.) was added to a solution of hydroxylamine prepared from hydroxylamine hydrochloride (14 g.) and potassium hydroxide (16·8 g.) in

TABLE I

NEW COMPOUNDS

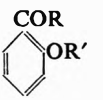


R	R′	Physical form	Melting point or boiling point, ° C.	Empirical formula	Required per cent			Found per cent		
					C	H	N	C	H	N
NH_2	2-$SC_5H_{11}^n$	Needles (*a*)	103	$C_{12}H_{17}NOS$	64·5	7·7	6·3	64·4	7·7	6·1
NH_2	3-$OC_5H_{11}^n$	Prisms (*b*)	117	$C_{12}H_{17}NO_2$	69·5	8·3	6·8	69·9	8·2	6·6
NH_2	3-$OC_6H_{13}^n$	Plates (*b*)	115	$C_{13}H_{19}NO_2$	70·5	8·6	6·3	70·7	8·5	6·1
NH_2	4-$OC_5H_{11}^n$	Needles (*b*)	158	$C_{12}H_{17}NO_2$	69·5	8·3	6·8	69·4	8·2	
NH_2	4-$OC_6H_{13}^n$	Prisms (*b*)	154	$C_{13}H_{19}NO_2$	70·5	8·6	6·3	70·7	8·4	6·2
OH	2-$OC_5H_{11}^n$	Prisms (*a*)	31–32	$C_{12}H_{16}O_2$	69·2	7·8		68·9	7·8	6·6
OH	2-$SC_5H_{11}^n$	Microcrystalline (*b*)	105	$C_{12}H_{16}O_2S$	64·3	7·2		64·5	7·3	
OMe	2-OBu^n	Liquid	118/17 mm.	$C_{12}H_{16}O_2$	69·2	7·7		69·4	7·8	
OMe	2-$OC_5H_{11}^n$	Liquid	110°/0·6 mm.	$C_{13}H_{18}O_2$	70·2	8·2		70·2	8·3	
OMe	3-$OC_5H_{11}^n$	Liquid	86/0·01 mm.	$C_{13}H_{18}O_2$	70·2	8·2		70·2	8·4	
OMe	3-$OC_6H_{13}^n$	Liquid	116/0·05 mm.	$C_{14}H_{20}O_2$	71·1	8·5		71·2	8·3	
OMe	4-$OC_6H_{13}^n$	Prisms (*a*)	22°	$C_{14}H_{20}O_2$	71·1	8·5		71·3	8·7	



R	Physical form	Melting point or boiling point, ° C.	Empirical formula	Required per cent			Found per cent		
				C	H	N	C	H	N
CONHOH	Matted needles (*c*)	87–88	$C_{12}H_{17}NO_3$	64·6	7·7	6·3	64·6	7·8	6·3
$CONHNH_2$	Needles (*d*)	32–33	$C_{12}H_{18}N_2O_2$	64·8	8·2	12·6	64·8	8·2	12·8
$CONHN{=}CHC_6H_5O$ (*f*)	Needles (*b*)	134–135	$C_{19}H_{21}N_2O_3$	69·9	6·8	8·6	70·0	6·9	8·5
CN	Liquid	126/1·0 mm.	$C_{12}H_{15}NO$	76·1	8·0	7·4	76·1	8·1	7·0
$CSNH_2$	Prisms (*b*)	80–81	$C_{12}H_{17}NOS$	64·5	7·7	6·3	64·2	7·8	6·3
$CH{=}NNHCONH_2$	Prisms (*b*)	182	$C_{13}H_{18}N_4O_2$	62·6	7·7	16·9	62·5	7·6	17·0
$CH{=}CHCO_2H$	Prisms (*e*)	98·5	$C_{14}H_{18}O_3$	71·8	7·7		71·7	7·7	
$CH{=}CHCONH_2$	Needles (*a*)	127	$C_{14}H_{19}NO_2$	72·1	8·2	6·0	71·8	8·1	5·9

Analyses by Drs. Weiler and Strauss and C. S. McRoe of these laboratories. M.ps. uncorrected.
(*a*) Recrystallised from light petroleum. (*b*) Recrystallised from ethanol.
(*c*) Recrystallised from aqueous ethanol. (*d*) Recrystallised from benzene/light petroleum.
(*e*) Recrystallised from ethyl acetate. (*f*) CHC_6H_5O = salicylidene.



R	R′	Boiling point °C.	Empirical formula	Required per cent		Found per cent	
				C	H	C	H
H	$C_6H_{13}^n$	108/0·1 mm.	$C_{13}H_{18}O_2$	75·7	8·8	75·7	8·8
Me	Bu^n	158°/19 mm.	$C_{13}H_{18}O_2$	75·0	8·4	74·9	8·7
Me	$C_6H_{13}^n$	170/17 mm.	$C_{13}H_{18}O_2$	75·7	8·8	75·9	8·7
Me	$C_6H_{13}^n$	106/0·2 mm.	$C_{14}H_{20}O_2$	76·3	9·2	76·1	9·2
Et	Bu^n	155/12 mm.	$C_{13}H_{18}O_2$	75·7	8·8	75·8	8·8
Et	$C_6H_{13}^n$	159/10 mm.	$C_{14}H_{20}O_2$	76·3	9·2	76·0	8·9
Et	$C_6H_{13}^n$	177/12 mm.	$C_{15}H_{22}O_2$	76·9	9·5	77·1	9·3

methanol (115 ml.) and left at room temperature for 12 hours. The solution was neutralised with acetic acid, and diluted with water (150 ml.) whereupon the product crystallised and was collected by filtration.

2-n-*Amyloxybenzhydrazide*. Methyl 2-*n*-amyloxybenzoate (10 g.) was heated under reflux with hydrazine hydrate (50 ml.) in ethanol (40 ml.) for 5 hours. The hydrazide was distilled, b.p. 163 to 164°/0·7 mm. and on cooling, solidified. The salicylidene derivative was formed from 2-*n*-amyloxybenzhydrazide and salicylaldehyde by the standard procedure.

2-n-*Amyloxybenzonitrile*. 2-*n*-Amyloxybenzamide (21·1 g.) was mixed with phosphorus pentoxide (14·2 g.) in dry xylene (250 ml.) and refluxed for 1 hour. The reaction mixture was then distilled, giving crude 2-*n*-amyloxybenzonitrile b.p. 178°/12 mm.

2-n-*Amyloxybenzthioamide*. 2-*n*-Amyloxybenzonitrile (20 g.) was dissolved in saturated ethanolic ammonium sulphide (120 ml.) and heated in a sealed tube at 100° for 5 hours. After evaporation of the solvent some solid separated and was collected and recrystallised to give 2-*n*-amyloxybenzthioamide. An attempt to prepare this compound by treating 2-*n*-amyloxybenzamide with phosphorus pentasulphide gave only 2-*n*-amyloxybenzonitrile in poor yield.

2-n-*Amyloxybenzaldehydesemicarbazone* was prepared from 2-*n*-amyloxybenzaldehyde and semicarbazide by the standard procedure.

2-n-*Amyloxycinnamic acid and* 2-n-*amyloxycinnamamide*. 2-*n*-Amyloxybenzaldehyde (19·2 g.) malonic acid (10·4 g.) and pyridine (15·8 g.) were mixed and heated at 100° for 4 hours. The mixture was poured into water and the oil extracted with chloroform. The chloroform solution was dried and evaporated, giving a residue of 2-*n*-amyloxycinnamic acid, which was purified by crystallisation. 2-*n*-Amyloxycinnamamide was prepared through the acid chloride by standard procedures.

Microbiological

Fungistatic test. Antifungal activity was assayed as previously described[1].

Fungicidal test. This is a modification, not previously reported, of the method of Golden and Oster[2].

1 sq. cm. plaques of Oxoid Membrane filter were inoculated from a culture of *Trichophyton mentagrophytes* and placed upon a plate of malt agar. After incubation at 28° for 10 days, the plaques covered with mycelium were removed from the agar, and placed in a solution of the fungicide in 25 per cent v/v acetone, 25 per cent v/v ethanol and 50 per cent distilled water. After an exposure of 1 hour the plaques were gently rinsed in 30 per cent aqueous acetone for 5 minutes and transferred to 20 ml. of Sabouraud broth for 1 hour. Finally, the plaques were placed mycelium downwards onto 10 per cent serum malt agar and incubated for 14 days at 28°.

In vivo *tests.* It is not possible to simulate human dermatomycoses in small laboratory animals. The method finally adopted was based upon that used by Frey[3].

Albino guinea pigs of approximately 250 g. were shaved over the flanks and the skin gently abraded by a scalpel blade without excessive bleeding. A suspension of 5×10^6/ml. spores of *T. mentagrophytes* in 50 per cent serum broth was then rubbed into the abraded area.

The infection normally follows a pattern; inflammation and scaling of the skin can be observed after 3 days, which increases until between the 4th and 10th day there is marked induration and exudation. The lesion then becomes heavily encrusted and spontaneous healing begins after 20 to 30 days. Baldness may persist for 50 days after infection.

Treatment was commenced 5 days after infection and continued for 8 days, assessment of infection was made visually at frequent intervals and where possible by microscopic and cultural examination. The period required for the regeneration of hair was taken as a further criterion in the assessment of a cure.

Toxicity

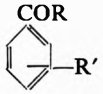
Acute toxicity was determined in albino mice using oral and intraperitoneal routes.

Irritation and sensitisation effects were examined by a modification of the method by Halpern[4], which depends on the increased capillary permeability caused by a local sensitivity reaction. 0·2 ml. of a 5 per cent ethanolic solution of the test compound was rubbed into the shaved backs of female albino guinea pigs once daily for ten days. Fourteen days after the last application the animals were given a shock dose of the compound (in a 20 per cent Tween 80) and injected intravenously with a solution of azovan blue. The appearance of a blue patch on the skin indicates a sensitivity reaction. Finally the animals were killed and the effect of the test compound on the isolated uterus was measured. A contraction of the uterus demonstrates a sensitivity reaction.

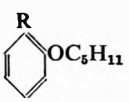
Patch tests, using 0·5 ml. of alcoholic gel covered by an occlusive dressing, were made on 93 human volunteers.

TABLE II

FUNGISTATIC ACTIVITIES



R	R′	Minimum inhibitory concentration, μg./ml.						
		Tm*	Tt	Tc	Tr	Ef	Ma	Index
NH_2	2-$OC_5H_{11}^n$ (7)	6	3	3	3	1·5	1·5	3
NH_2	2-$SC_5H_{11}^n$	>50	>50	>50	>50	>50	>50	>50
NH_2	2-$OC_6H_{13}^n$ (7)	12·5	3	3	3	3	1·5	4·3
NH_2	3-$OC_5H_{11}^n$	>50	25	25	25	25	12·5	>27
NH_2	3-$OC_6H_{13}^n$	>50	>50	>50	>50	>50	>50	>50
NH_2	4-$OC_5H_{11}^n$	>50	>50	>50	>50	>50	>50	>50
NH_2	4-$OC_6H_{13}^n$	>50	>50	>50	>50	>50	>50	>50
OH	2-O Bu^n (8)	>50	>50	>50	>50	>50	>50	>50
OH	2-$OC_5H_{11}^n$	>50	50	25	50	25	25	>38
OH	2-$SC_5H_{11}^n$	>50	>50	>50	>50	>50	>50	>50
OH	3-$OC_5H_{11}^n$ (8)	50	25	25	25	25	6	26
OH	3-$OC_6H_{13}^n$ (9)	>50	25	25	>50	25	25	>33
OH	4-$OC_5H_{11}^n$ (10)	50	25	25	>50	50	25	>38
OH	4-$OC_6H_{13}^n$ (10)	>50	50	50	>50	50	50	>50
OMe	2-O Bu^n	50	25	25	25	50	25	33
OMe	2-$OC_5H_{11}^n$	50	25	25	12·5	25	12·5	25
OMe	3-$OC_5H_{11}^n$	>50	25	25	25	50	25	>33
OMe	3-$OC_6H_{11}^n$	>50	>50	>50	>50	50	50	>50
OMe	4-$OC_5H_{11}^n$ (11)	>50	>50	50	25	>50	25	>42
OMe	4-$OC_6H_{13}^n$	>50	>50	>50	>50	>50	>50	>50



	R	Tm	Tt	Tc	Tr	Ef	Ma	Index
	CONHOH	50	25	12·5	6	12·5	12·5	20
	$CONHNH_2$	>50	>50	>50	>50	>50	>50	>50
	$CONHN{=}CHC_6H_4O$†	>50	>50	>50	>50	>50	>50	>50
	CN	50	50	50	50	>50	25	>46
	$CSNH_2$	25	12·5	6	6	6	1·5	9·5
	$CH = NNHCONH_2$	>50	12·5	12·5	25	25	6	>22
	$CH = CHCO_2H$	50	25	25	50	25	12·5	23
	$CH = CHCONH_2$	>50	>50	>50	>50	>50	>50	>50

* *Key*—Tm = *T. mentagrophytes*; Tt = *T. tonsurans*; Tc = *T. concentricum*; Tr = *T. rubrum*; Ef = *E. floccosum*; Ma = *M. audouini*.
† CHC_6H_4O = salicylidene.

R	R'	Tm	Tt	Tc	Tr	Ef	Ma	Index
H	H	>50	>50	>50	>50	>50	>50	>50
H	Bu^n (12)	50	25	25	50	12·5	12·5	29
H	$C_5H_{11}^n$ (13)	25	9·3	12·5	12·5	12·5	9·3	13·5
H	$C_6H_{13}^n$	25	12·5	25	25	25	25	23
Me	H	>50	>50	>50	>50	>50	>50	>50
Me	Bu^n	25	25	25	25	12·5	6	20
Me	$C_5H_{11}^n$	18·5	18·5	9·3	18·5	9·3	4·6	13·1
Me	$C_6H_{13}^n$	50	25	12·5	12·5	12·5	6	20
Et	H	>50	>50	>50	>50	>50	>50	>50
Et	Bu^n	50	25	25	25	12·5	6	24
Et	$C_5H_{11}^n$	50	25	6	50	6	3	23
Et	$C_6H_{13}^n$	>50	>50	>50	>50	>50	>50	>50

Results

Microbiological

Fungistatic tests. The minimum inhibitory concentration of 40 compounds against six representative dermatophytes are recorded in Table II.

Antagonism. 2-*n*-Amyloxyacetophenone and 2-*n*-amyloxybenzamide have been screened in the presence of 10 per cent whole blood, 10 per cent serum, 8 per cent keratin hydrolysate, 2·5 per cent by weight of hair, 0·1 per cent soap, 0·02 per cent of sodium lauryl sulphate, 0·025 per cent of an artificial sebum mixture[5], and 0·01 per cent *p*-aminobenzoic acid. 10 per cent serum halved the activity of 2-*n*-amyloxyacetophenone and reduced

TABLE III
FUNGICIDAL ACTIVITY

Compound	Exposure period	Concentration per cent	
		1·0	0·1
2-*n*-Amyloxybenzamide	1 hr.	–	–
2-*n*-Amyloxyacetophenone ..	1 hr.	–	–
Salicylanilide	1 hr.	–	–
Control-solvent only	1 hr.	+	
		Growth after 7 days incubation	

– No growth
+ Growth
} on subculture medium at 14 days

the activity of 2-*n*-amyloxybenzamide to one third. Sodium lauryl sulphate halved the activity of 2-*n*-amyloxybenzamide but had no effect on 2-*n*-amyloxyacetophenone. The remaining substances tested did not significantly affect the minimum inhibitory concentrations obtained for the two compounds.

Fungicidal tests. Results of fungicidal tests are given in Table III.

In vivo *tests.* *In vivo* test results are shown in Table IV.

Experiment 1 shows the effect of 2-*n*-amyloxybenzamide and salicylic acid, used as a keratolytic agent, singly or in combination, using a water-in-oil emulsion base. Experiment 2 shows the effect of 2-*n*-amyloxybenzamide, 2-*n*-amyloxyacetophenone and salicylic acid in different

TABLE IV

IN VIVO FUNGICIDAL ACTIVITY USING GUINEA PIGS

Group	No. of animals	Treatment	No. cured at 18 days	Time taken for hair regrowth
Exper. 1 1	5	3 per cent AB* in water in oil emulsion base	None	>47 days
2	5	3 per cent AB, 2 per cent SA in water in oil emulsion base	4	37 days
3	5	2 per cent SA, in water in oil emulsion base	2	47 days
4	5	Controls base only	None	47 days
Exper. 2 1	5	2 per cent AA, 2 per cent AB and 1 per cent SA in alcoholic gel	3	45 days
2	5	2 per cent AA and 1 per cent SA in alcoholic gel	1	>45 days
3	5	2 per cent AB and 1 per cent SA in alcoholic gel	1 and 1 improved	>45 days
4	5	2 per cent AA and 2 per cent AB in alcoholic gel	3	>45 days
5	5	2 per cent AA in alcoholic gel	1 improved	45 days
6	5	Controls gel base only in alcoholic gel	1 improved	>45 days

Exper. 3			Progress of cure at day				Animals culture positive at day				Hair regrowth
			10	14	17	21	10	14	17	21	
1	6	2 per cent AA, 2 per cent AB, 1 per cent SA in alcoholic gel base	22	16	5	0	1	0	1	Insufficient material	36 days
2	6	2 per cent AA, 2 per cent AB, 1 per cent SA in water in oil emulsion	7	12	5	1	2	0	1		>36 days
3	6	2 per cent AA, 2 per cent AB, 10 per cent U in Carbopol 934 gel	21	9	2	1	0	1	1		>36 days
4	6	2 per cent AA, 2 per cent AB, 1 per cent SA in P.E.G. base	7	6	3	0	2	0	1		36 days
5	6	0·5 per cent DM(DE)B in P.E.G. base	17	17	7	2	6	1	4		<36 days
6	6	Untreated	24	13	6	2	3	4	4		>36 days

* *Key*—AA = 2-*n*-amyloxyacetophenone; AB = 2-*n*-amyloxybenzamide; U = urea; SA = Salicylic acid and DM(DE)B = 2-dimethylamino-6-(β-diethylaminoethoxy)benzthiazole.
P.E.G. = Polyethylene glycol.

combinations in an alcoholic gel base. Experiment 3 records the results obtained using the two active ingredients incorporated with a keratolytic in a series of different bases.

In this last experiment a scoring system was used and cultures of skin scales taken to determine the presence or absence of fungi. This was found necessary when it became clear that a single assessment of "cure"

at a fixed time during the test gave an incomplete picture of the efficacy of a preparation. A parallel experience is cited by Frey[3]. The scoring system involved a visual estimate of the state of infection, the observer being unaware of the nature of the treatment. Values of 0, 1, 2, 3 and 4 were used to indicate increasing severity of infection, i.e., 0 indicates complete cure and 4, a heavy infection. The figures in Table IV, Experiment 3, represent the total score for the group. The number of positive cultures, and the time for hair re-growth gives additional information concerning the value of the treatment.

Toxicity

Acute Toxicity in mice. Oral LD50 2-*n*-amyloxybenzamide 4·7 g./kg.[6], 2-*n*-amyloxyacetophenone 6·0 g./kg. Intraperitoneal LD50 0·9 g./kg. and 0·6 g./kg., respectively.

Irritation and sensitisation effects. With 2-*n*-amyloxybenzamide and 2-*n*-amyloxyacetophenone no reactions were recorded when each compound was tested in 20 guinea pigs using the Halpern method. Similarly no reactions were recorded by the isolated uterus technique, from which it is concluded that neither substance is likely to prove a sensitising agent. With human volunteers no reactions were recorded in a patch test to a mixture of 2-*n*-amyloxybenzamide and 2-*n*-amyloxyacetophenone, applied in an alcoholic gel base. Samples of a product containing 2 per cent 2-*n*-amyloxybenzamide, 2 per cent 2-*n*-amyloxyacetophenone and 1 per cent salicylic acid have been used clinically in 60 patients and have given no adverse reactions.

Discussion

It has been previously shown[1] that in a series of 2-alkoxybenzamides peak activity occurred with the amyloxy and hexyloxy compounds and in the work now reported, alkoxy substitution was confined to these groups and the effects of alteration of the nature and position of the other substituent have been examined. To study the effect of alteration in relative position of the two substituents, a group of 3-, and 4-alkoxybenzamides were examined and shown to be of very low activity compared with the 2-isomers. Replacement of the amide group by methoxycarbonyl caused a reduction in activity but here also the 2-isomers were the most active, with highest activity occurring in the amyloxy compound. The corresponding carboxylic acids were of low activity and showed no clear relationship between structure and activity.

In view of previous reports[14] that the replacement of oxygen by sulphur increases antifungal activity, 2-*n*-amylmercaptobenzamide and 2-*n*-amyloxybenzthioamide were examined. The replacement of ether oxygen by sulphur led to complete loss of activity, and similar replacement of the carbonyl oxygen, to a slight loss of activity, although this latter compound was the most active of all those reported in this paper. 2-*n*-Amyloxybenzhydrazide and its salicylidene derivatives were inactive, but the corresponding hydroxamic acid retained some activity, although of a reduced order. Urbanski and co-workers[15,16] have recently reported on the use of

salicylhydroxamic acid, orally, in the clinical treatment of dermatophyte infections, and on the powerful *in vitro* fungistatic effect of a series of halogenated phenoxyacethydroxamic acids.

The replacement of the amide group of 2-*n*-amyloxybenzamide by other carbonyl containing radicals has yielded interesting results. 2-Alkoxybenzaldehydes, acetophenones and propiophenones all showed pronounced activity, and in this group of compounds peak activity was again found with the amyloxy-derivatives. 2-*n*-Amyloxybenzaldehyde and 2-*n*-amyloxyacetophenone were the most active of this group, and the ketone was selected for further more detailed study in view of its greater stability.

It seems that within the limited class of compounds studied, the necessary structural requirements for antifungal activity are a benzene ring bearing an *n*-amyloxy-group *ortho* to a carbonyl radical, although exceptions to this rule were noted (2-*n*-amyloxybenzoic acid and -benzhydrazide).

The fungicidal test results (Table III) show 2-*n*-amyloxybenzamide, 2-*n*-amyloxyacetophenone and salicylanilide to be fungicidal after an exposure of 1 hour to concentrations of 0·1 per cent in water : ethanol : acetone mixture. We previously reported[1] 2-*n*-amyloxybenzamide to be fungicidal only after 24 hours exposure to 0·1 per cent concentration of the compound in Dubos broth. The difference between these two results is probably due to the sparingly soluble nature of 2-*n*-amyloxybenzamide in aqueous media.

All available methods for the assessment of antifungal activity in experimental animals suffer from certain disadvantages, such as the difference between animal and human skin, the differences in pathogenesis, and the spontaneous regression which occurs with dermatophyte infections in animals. Calves[17,18] and guinea pigs[3,19] have been suggested as suitable experimental animals. Guinea pigs were chosen because of availability and the regular and reproducible nature of the infection produced in such animals, although the lesions differ in many important respects from those occurring in man.

Experiment 1 (Table IV) showed 2-*n*-amyloxybenzamide to be inactive when used alone in a water-in-oil emulsion base, but when salicylic acid was added as a keratolytic a marked effect was shown. Salicylic acid alone produced some curative action but was considerably less effective than the mixture.

The discovery of the antifungal effect of 2-*n*-amyloxyacetophenone, an oil, together with its powerful solvent properties for 2-*n*-amyloxybenzamide led us to investigate the effect of combinations of these two substances with and without salicylic acid in an alcoholic gel base. Experiment 2 (Table IV) showed these to be an effective combination.

Bushby and Stewart[20] emphasised the importance of the pharmaceutical base in which antifungal agents are administered. Experiment 3 was designed to show the effect of different bases on the antifungal effect of a standard mixture of the two active substances combined with a keratolytic. Experience with earlier tests led us to the conclusion that therapeutic effect should not be assessed at one time only, for the infection shows

three phases: an initial inflammatory phase, then a period when isolation of the fungus is readily accomplished, which is followed by regression of infection and hair regrowth. In this last test, therefore, the state of infection was visually assessed at various times after infection, and in addition, scrapings from the lesions were examined both microscopically and by culture for the presence of fungi. The results show that the duration of the inflammatory phase was markedly shortened by water-in-oil emulsion, and polyethylene glycol based preparations of 2-*n*-amyloxybenzamide and 2-*n*-amyloxyacetophenone. The same ingredients in an alcoholic gel base, although showing little effect on the inflammatory phase, nevertheless considerably reduced the number of positive cultures, and the time necessary for hair regrowth.

These results, together with the low toxicity of the active ingredients and their lack of sensitising or irritant properties, suggested that suitable formulations of these compounds might be of value in the treatment of human dermatophyte infections.

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References

1. Coates, Drain, Kerridge, Macrae and Tattersall, *J. Pharm. Pharmacol.*, 1957, **9**, 855.
2. Golden and Oster, *J. Amer. pharm. Ass., Sci. Ed.*, 1947, **36**, 359.
3. Frey, *Dermatologica*, 1953, **107**, 88.
4. Halpern, Communication to the British Pharmacological Society, 1953, Jan.
5. Klarmann, *American Perfumer*, 1948, July.
6. Bavin, Macrae, Seymour and Waterhouse, *J. Pharm. Pharmacol.*, 1952, **4**, 872.
7. British Patent No. 726,786.
8. Pierce, Salsbury and Frederickson, *J. Amer. chem. Soc.*, 1942, **64**, 1693.
9. Jones, *J. chem. Soc.*, 1943, 430.
10. Jones, *ibid.*, 1935, 1874.
11. Cavill and Gibson, *J. Soc. Chem. Ind.* (*Lond.*), 1947, **66**, 274.
12. Buu-Hoi, *C.R. Acad. Sci., Paris*, 1945, **221**, 202.
13. Katz, Karger, Schroeder and Cohen, *J. org. Chem.*, 1953, **18**, 1380.
14. Klöpping, Thesis, Chemical constitution and Antifungal action of Sulphur compounds, Schotanus and Jens, Utrecht, 1951, pp. 66–68.
15. Alkiewicz, Eckstein, Halweg, Krakowka, and Urbanski, *Nature, Lond.*, 1957, **180**, 1204.
16. Eckstein, Halweg, Krakowka and Urbanski, *Bull. Acad. Polon. Sci.*, 1958, **6**, 235.
17. Sellers, *Analyst*, 1956, **81**, 77.
18. Sellers, Sinclair and LaTouche, *Vet. Rec.*, 1956, **68**, 729.
19. Jadassohn and Pfanner, *Dermatologica*, 1945, **91**, 46.
20. Bushby and Stewart, *Brit. J. Dermat. Syph.*, 1949, **61**, 315.

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

SODIUM NOVOBIOCIN: STABILITY ASPECTS

By M. J. Busse, K. A. Lees and V. J. Vergine

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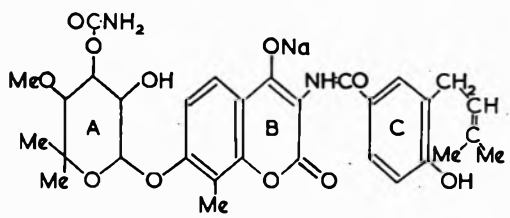
The stability of monosodium novobiocin has been examined. The solid antibiotic has been shown to be sensitive to light, but if properly protected, to be stable for a period of 2 years at 20°. In aqueous solution and suspension it is affected by increase of temperature, pH and certain heavy metals. Phosphate, and possibly sulphate, also cause decomposition. Concentrated solutions of novobiocin become coloured, particularly above 20° and in the presence of air. Light has little effect on potency but irradiation with ultra-violet light accelerates potency loss in solution. There appears to be no difference in the potency stability of three different batches of sodium novobiocin. Ultra-violet spectrophotometry cannot be used as a method of assay.

Novobiocin is a dibasic acidic antibiotic produced by the actinomycete *Streptomyces niveus*. In neutral to slightly alkaline solutions it exists as the monosodium salt (I), the disodium salt being formed in strongly alkaline solution.



(I)

The acidic form of novobiocin has been reported almost insoluble in water, but it is soluble at pH values between 7·5 and 9·5[1]. There are only few references in the literature to the stability of novobiocin. Stability in aqueous solution is said to be a function of pH and temperature; dilute solutions at pH 2 are stable at 24°, but a half-life of about 60 days is observed at pH values between 7 and 10. A 10 per cent solution of sodium novobiocin at pH 7·5 is said to have a half-life of 30 days at 24° and of several months at 4°. Mention is made of the light sensitivity of the dry antibiotic[2].

Methods

A large plate microbiological method was employed for determinations of potency. The unit of activity of novobiocin is defined as one microgram of pure novobiocin acid. The designs used were 8×8 Quasi-Latin squares capable of yielding results with a standard error of ± 7 per cent ($P = 0{\cdot}95$) from the means of duplicate assays by separate operators. The test organism was *B. subtilis* (MB.32) seeded into neutral agar medium at pH 7·0 and incubated for 18 hours at 30°. The reference standard was sodium novobiocin of high potency and low moisture content, previously

standardised against a sample of novobiocin lodged with the Standards Department of the M.R.C., Mill Hill, for the purpose of creating a British National Standard. For assay, samples and standards were diluted in a phosphate buffer solution at pH 7·5 (0·98 per cent disodium hydrogen phosphate, 0·19 per cent potassium dihydrogen phosphate) to 10 and 5 u./ml., concentrations suitable for plating out. Colours were measured with the "Eel" Portable Colorimeter.

Experimental and Results

Stability of Solid Monosodium Novobiocin

Dry solid monosodium novobiocin (moisture content 3·0 per cent, by loss under reduced pressure at 60°) was stored at 4°, room temperature, in the dark or exposed to light, at 37° or at 50°. The results revealed no potency loss after 2 years storage at 4°, 20° or 37° in the dark. At 50°

TABLE I

EFFECTS OF CONCENTRATION AND TEMPERATURE ON POTENCY OF STORED MONOSODIUM NOVOBIOCIN SOLUTIONS

Temperature °C.	Potency u./ml.					
	Initial	4 days	2 weeks	4 weeks	10 weeks	16 weeks
4	315,000				300,000*	
20	315,000		Solidified			
37	315,000	240,000	125,000			
4	68,000				61,000	
20	68,000		57,500	55,000		46,000
37	68,000	52,000	40,000	30,000		
4	13,800				13,000	
20	13,800		12,500	12,500		10,200
37	13,800	12,000	9,500	8,000		
4	2,500				2,450	
20	2,500		2,550	2,400		2,230
37	2,500	2,450	1,950	1,500		
4	530				505	
20	530		520	510		454
37	530	490	410	365		
4	110				100	
20	110		95	95		94·5
37	110	96	80	70		

* Solidified

approximately 10 per cent potency loss was recorded after 2 years. The samples exposed to light showed potency loss, regular mixing resulting in potency loss from 935 u./mg. to 680 u./mg.

A sample of solid sodium novobiocin, moisture content 2·7 per cent, was dried under reduced pressure at 50° for 3 hours to give material of moisture content 1·1 per cent. Another sample of the same batch was kept at 75 per cent relative humidity and 37° for 48 hours, after which the moisture content was 11 per cent. This material was blended with some of the sample containing 2·7 per cent moisture to give a product with moisture content of 5·8 per cent. The three samples of 1·1, 2·7 and 5·8 per cent moisture contents were heated in closed containers at 100° for 16 hours and assayed when the potency results obtained were 890, 740

and 685 u./mg. respectively. No change in the visible appearance of any sample was noticeable after heating.

Stability of Aqueous Solutions and Suspensions

Novobiocin is soluble in water at alkaline pH values, but is precipitated as the free acid in water at acid pH values. All solutions, unless otherwise stated, were prepared in distilled water and all samples, unless otherwise stated, were stored in the dark.

Stability at different concentrations. Solutions at the six concentrations levels were prepared: 312,500, 62,500, 12,500, 2,500, 500, and 100 u./ml. They were stored in 20 ml. ampoules at 4°, 20° or 37° and were assayed for potency, the colour development also being measured at intervals. The potency results are shown in Table I. A graph was drawn plotting the percentage potencies of the solutions stored at 37° against time. The 60 per cent, 70 per cent and 80 per cent lives for each concentration read from this graph were plotted against log concentration, as shown in Figure 1.

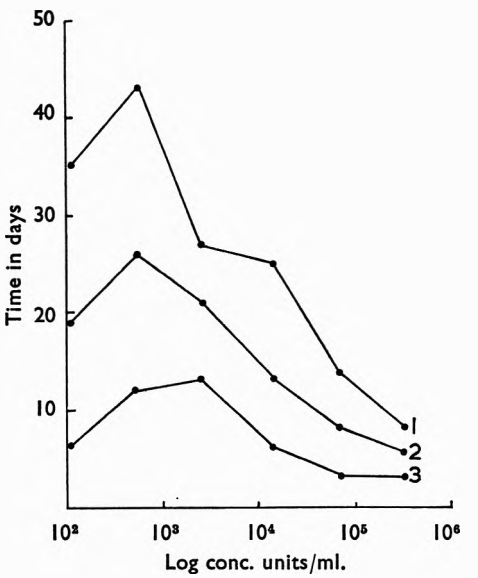


FIG. 1. Effect of concentration on the potency of sodium novobiocin solutions stored at 37°. 1. 60 per cent potency. 2. 70 per cent potency. 3. 80 per cent potency.

At a concentration of 2500 u./ml., or less there was no visually significant colour development at either 20° or 37°. A solution containing 12,500 u./ml., gave slight colour development when stored at 37° for six weeks but solutions of 62,500 and 312,500 u./ml. acquired appreciable colour after only 7 days storage. There was negligible colour development at 4° except in the 31·25 per cent solution, for which the Eel colorimeter reading (OB.2 filter) rose from 25 to 32 in 7 days.

Effect of pH value on stability. Sodium novobiocin as 5 per cent w/v solutions and suspensions in water at pH's 3·3, 5·0, 7·0 (suspensions) or 9·0 (solutions) was filled into 20 ml. ampoules, stored at 37° and assayed at intervals. The results are shown in Table II. In this series the pH values 3·3, 5·0 and 7·0 were obtained by means of phosphate-citrate buffers and pH 9·0 by means of a glycine-sodium hydroxide buffer. A similar series was prepared for room temperature (20°) storage, the only difference being that the solution at pH 9·0 was prepared with a sodium borate buffer. The results are shown in Table II.

Effect of air and antoxidants. We have already shown that sodium novobiocin solutions develop colour on storage; because of this the effect of air and the action of antoxidants on stability of potency and on colour development in sodium novobiocin solutions were investigated.

The antoxidants selected were sodium formaldehyde sulphoxylate (S.F.S.) and sodium metabisulphite. It was found that solutions of sodium novobiocin (50,000 u./ml.) containing 0·2 per cent S.F.S. formed a

TABLE II

EFFECTS OF pH AND TEMPERATURE ON POTENCY OF STORED NOVOBIOCIN SOLUTIONS AND SUSPENSIONS

pH	Temperature °C.	Potency u./ml.						
		Initial	6 days	21 days	6 weeks	12 weeks	24 weeks	16 months
3·3	20	46,900	47,000			55,000	47,000	49,700
	37	45,000	47,700	43,700				
5·0	20	43,800	36,000		53,500	51,200	48,000	47,000
	37	49,800	44,500	28,800				
7·0	20	47,200	48,300		43,200	34,800	25,000	
	37	45,700	30,400	9,500				
9·0	20	45,100	36,000		21,500	800		
	37	44,600	19,300	5,100				

precipitate and became cloudy within a week at room temperature or 37°. Nevertheless, despite the cloudiness, the solutions containing S.F.S. remained considerably lighter in colour, both at room temperature and at 37°, than the corresponding control (without S.F.S.) both solutions being stored in the presence of a large volume of air.

TABLE III

POTENCY OF SODIUM NOVOBIOCIN SOLUTIONS AND SODIUM NOVOBIOCIN SOLUTIONS CONTAINING 0·25 PER CENT SODIUM METABISULPHITE STORED IN AMPOULES SEALED UNDER NITROGEN AND IN 4-OZ. BOTTLES WITH A LARGE AIR SPACE

Solution	37° C.			20° C.
	Initial potency	14 days, u./ml.	4 weeks, u./ml.	4 weeks, u./ml.
Control (nitrogen)	53,000	35,000 (66 per cent)	28,900 (54·5 per cent)	40,300 (76 per cent)
Control (large air space)	53,000	33,900 (63 per cent)	19,900 (38 per cent)	45,300 (85 per cent)
Metabisulphite (nitrogen)	49,500	28,100 (56·5 per cent)	19,200 (39 per cent)	39,200 (79 per cent)
Metabisulphite (large air space) ..	49,500	23,900 (48 per cent)	<2,000	33,200 (67 per cent)

Two solutions of sodium novobiocin at a concentration of 50,000 u./ml. were prepared, and 0·25 per cent sodium metabisulphite was added to one of them. The latter solution was prepared by dissolving the sodium metabisulphite in a small quantity of water and adding NaOH solution to bring the pH to 7·0. This solution was then added to the sodium novobiocin solution, adjusted to the same pH value as the control (pH 8) with further NaOH and made up to volume. The

sodium metabisulphite was neutralised in this way to prevent precipitation of novobiocin free acid. Both these solutions were stored in 20 ml. ampoules sealed under nitrogen, and also in 4-oz. bottles with a 70: 30 air/solution ratio. The potency stability of these solutions at 20° and 37° is shown in Table III. The colours of the four solutions stored under nitrogen, or with a large air space, or containing 0·25 per cent sodium metabisulphite in the presence of either nitrogen or air, gave Eel colorimeter readings (OB.2 filter) of 16, 32, 7·5 and 41 respectively after 4 weeks' storage at 37°, the initial colour reading being 4·0.

Effect of buffer. The effect on the stability of sodium novobiocin solutions (50,000 u./ml.) of 2 per cent of disodium hydrogen phosphate,

TABLE IV

POTENCY OF SOLUTIONS OF THREE BATCHES OF SODIUM NOVOBIOCIN STORED AT ROOM TEMPERATURE OR 37°

Batch	Temperature °C.	Potency, u./ml.				
		Initial potency	4 days	1 week	2 weeks	4 weeks
A	R.T.	160,000			140,000	120,000
	37	160,000	110,000	95,000	75,000	
	37	185		155	125	115
B	R.T.	155,000			135,000	120,000
	37	155,000	115,000	85,000	65,000	
	37	175		105	90	95
C	R.T.	140,000			125,000	110,000
	37	140,000	110,000	85,000	70,000	
	37	150		100	95	90

sodium citrate or glycine was investigated and compared with that of a control. All the solutions, including the control, had pH values of 8·0, the one containing phosphate being adjusted to this pH value with 0·003 per cent v/v orthophosphoric acid. The potency results after 4 weeks' storage at 37° were 60, 25 and 61 per cent of the initial potencies for the control, sodium phosphate buffer and sodium citrate buffer solutions respectively. The solution containing glycine precipitated after 7 days' storage at 37°. The solutions developed colour and after 4 weeks' storage at 37° recorded Eel colorimeter readings (OB.2 filter) of 21, 27 and 22 for the control, phosphate and citrate buffer solutions respectively, the initial colour reading being 4·0.

Effect of visible and ultra-violet light on stability. Suspensions and solutions over a range of pH values were prepared, and, after storage in the dark or exposure to daylight, some being left undisturbed and others shaken daily, were assayed at intervals. A similar test was carried out on samples exposed to ultra-violet radiation from a 3·75 kw. lamp at 2 to 3 feet from the samples. Suspensions prepared at pH 3·6, 4·6, and 6·3 retained full potency for 6 months at 20° both when stored in the dark and when exposed to light and regularly shaken. Suspensions at pH 7·0 and solutions at pH 7·5 showed a loss in potency over the same period of approximately 40 per cent, there being no significant difference between samples stored in the dark and exposed to light. Suspensions at pH 3·8 and 5·4

exposed to ultra-violet light and regularly shaken showed no loss after 18 days whilst solutions at pH 7·8 showed approximately 25 per cent loss in activity over the same period, the control solution stored in the dark remaining stable.

Effect of heavy metals. The effect of five metals was investigated; copper, iron, nickel, zinc and lead. It was found necessary to add the solution of copper sulphate, ferric chloride, nickel sulphate, zinc sulphate or lead nitrate to suspensions of novobiocin (pH 6·3) as a precipitate formed when any of the metal salts were added to a sodium novobiocin solution at slightly alkaline pH. Each metal salt was used at a concentration of 20 p.p.m., calculated as metal. The concentration of sodium novobiocin was 2·5 per cent. None of the suspensions lost significant potency after 12 months' storage at 20°. The potency results after 3

TABLE V

COLOUR DEVELOPMENT OF SOLUTIONS OF THREE BATCHES OF SODIUM NOVOBIOCIN STORED AT ROOM TEMPERATURE OR 37° (EEL COLORIMETER—OB.2 FILTER)

Batch	Temperature, °C.	Initial colour	4 days	1 week	2 weeks	4 weeks
A	R.T.	10·5			15·0	18·0
	37	10·5	22·0	27·5	36·0	33·0
B	R.T.	9·5			13·0	15·0
	37	9·5	18·0	22·0	29·0	25·0
C	R.T.	20·0			26·0	30·0
	37	20·0	34·0	70·0	61·0	48·0

weeks' storage at 37° were 70, 64, 62, 32, 33 and 48 per cent of the initial potency for the control suspension, the suspension containing copper, iron, nickel, zinc and lead respectively.

Comparative stability of different batches of sodium novobiocin. The comparative stability of solutions of three different batches of monosodium novobiocin has been examined. The results are shown in Table IV. The colours of the stored solutions prepared from the three batches are recorded in Table V.

Solutions of sodium novobiocin that have lost most of their biological potency possess a distinct odour of ammonia. Such a solution, at a concentration of 50,000 u./ml., which had been stored for 4 weeks at 37°, was assayed both microbiologically, giving a figure of 29,000 u./ml., and spectrophotometrically, giving a figure of 49,000 u./ml.

DISCUSSION

Solid sodium novobiocin would appear to be reasonably stable for 2 years at temperatures up to 37° when protected from light. Exposure to light produced a dark yellow colour on the exposed surface, whereas the unexposed material below maintained the normal "off-white" colour. Mixing caused a significant drop in potency of exposed powder and the production of a uniform pale yellow colour throughout the bulk. It would appear that no special temperature precautions are required for the storage of solid sodium novobiocin, but it is important to protect from

light. An increase in moisture content renders solid sodium novobiocin less stable.

The potency results shown in Table I reveal the relatively lower stability of the concentrated solutions of sodium novobiocin. Figure 1 indicates that stability gradually increases as concentration decreases, the optimum concentration possibly being in the range 500 to 2500 u./ml., below which stability decreases. Further work is needed before this peak for optimum stability can be accepted.

The differing stabilities of the solutions at different concentrations might possibly be explained by the pH values of the solutions which were:

Concentration	pH
31·25 per cent ..	8·2
6·25 per cent ..	7·7
1·25 per cent ..	7·3
0·25 per cent ..	6·8
0·05 per cent ..	6·6
0·01 per cent ..	6·5

The lower stability at the higher concentrations might therefore be explained by the demonstration of the effects of hydrogen ion concentration on stability. From the results of colour development it is evident that solutions of concentration 6·25 per cent or less, stored under conditions that maintain potency also remain satisfactory in colour. Dilute solutions (0·25 per cent and less) show no visible colour development even though stored under conditions resulting in loss of potency.

The results in Table II indicate clearly that the stability of novobiocin in an aqueous system decreases with increase in pH value. This demonstration of lowered stability with increase in pH value made it useful to know how much of the antibiotic present in suspensions of free novobiocin acid is in true solution at various pH values. Spectrophotometry of filtrates of suspensions of free novobiocin acid of concentration 50,000 u./ml. at pH values 7·0, 6·0, 5·0, 4·0 and 3·0 revealed novobiocin contents of 14,500, 850, 350, less than 50 u./ml., and zero respectively.

At room temperature no apparent effect of metals on the stability of novobiocin has been demonstrated, but at 37° it appears that nickel, zinc or lead salts may have some action in accelerating potency loss.

No significant differences in the potency stability of solutions prepared from three different batches of sodium novobiocin has been detected however the extent to which colour develops on storage depends on the initial colour of the solution.

Although sodium novobiocin solutions are not sufficiently stable at pharmaceutical concentrations (say 0·1 per cent and above) to meet the requirements for shelf-life of a pharmaceutical product, suspensions of free novobiocin acid would meet these requirements (see Table II). However, the results in Table I indicate that solutions of concentration 6·25 per cent and less would be stable for several months if stored in a refrigerator and for about a month at room temperature.

Hoeksema and others[2] mention that a 10 per cent solution of novobiocin has a half-life of 30 days at 24°. Our results indicate much greater stability than this, for our solutions of concentration 6·25 per cent (see Table II) and 15 per cent (see Table IV) exhibited 80 per cent potency after 4 weeks' storage at room temperature (approximately 20°). We have also shown in preliminary work that a 10 per cent solution has a half-life of about 14 weeks at room temperature.

Novobiocin as a dry solid (sodium salt) is sensitive to visible light, but this sensitivity has not been clearly demonstrated for solutions and suspensions of novobiocin; on the other hand ultra-violet light causes potency losses in solutions, with apparently no effect on suspensions of free novobiocin acid under the particular conditions of the test.

Sodium metabisulphite appears to stabilise the colour of sodium novobiocin solutions in the absence of air, but in the presence of air it has the opposite effect; it causes loss of potency both in presence and absence of air. It is possible that the presence of sulphate arising from the oxidation of sodium metabisulphite might affect sodium novobiocin solutions in a manner similar to that which we have demonstrated for sodium phosphate.

Acknowledgement. We wish to thank Mr. J. S. Simpson, Analytical Department, Glaxo Laboratories Ltd., for the microbiological assays.

REFERENCES

1. Frost, Valiant, McClelland, Solotorovsky and Cuckler, *Antibiotics Annual*, 1955–6, 918.
2. Hoeksema, Bergy, Jackson, Shell, Hinman, Fonken, Boyack, Garon, Ford, Devries and Crum, *Antibiotics and Chemotherapy*, 1956, 6, 146.

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

Short Communication

"FOOTPRINTS" IN ADSORBENTS

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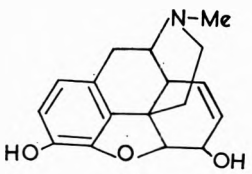
Received June 1, 1959

The investigation of the possible preparation of three dimensional footprints in adsorbents had a two-fold objective, namely, to provide a new method for the elucidation of the configuration of molecules and to try to produce models of biological receptor surfaces. A three-dimensional approach to the consideration of biological receptor surfaces and medicinal chemistry seemed imperative since the majority of chemical substances formed or broken down in normal metabolic processes are optically active.

A preliminary report of the preparation and application of stereoselective adsorbents has already been given[1]. The method involves the preparation of "footprints" of the desired three-dimensional arrangement in silica gel by forming the gel in the presence of the reference molecule, drying and reducing to a powder and extracting the reference molecule from the surface layers of the adsorbent. These adsorbents distinguish stereoisomers of the same, from those of different configuration providing the molecule used to prepare the configurational footprint is not too dissimilar in structure from the molecules to be adsorbed. Very good discrimination was obtained using cinchona alkaloids as reference molecules[1].

The presence of configurational footprints to account for the increased uptake by stereoselective, as compared with blank adsorbents (prepared in the absence of the reference molecule), rather than an explanation based upon increased surface area of the former adsorbents is indicated by the stereoselectivity of the adsorbents, for example, a gel with a quinine footprint will adsorb quinine and cinchonidine (same configuration) more readily than quinidine and cinchonine (different configurations) but a quinidine adsorbent will adsorb quinidine and cinchonine more readily than quinine and cinchonidine. Also when molecules greatly dissimilar from the molecule used to make the footprint are adsorbed the specificity is decreased, for example, a gel with the quinine footprint will adsorb quinine, and to a lesser extent quinidine, much more readily than will a blank gel, but this difference is greatly diminished using 5-aminoacridine. Further support for the physical reality of footprints derives from storage experiment, for example, the storage of a blank gel and one with a quinine footprint for 6 months in a refrigerator gives no change in the adsorbent properties of either, but storage at 37° gives some loss of selectivity of stereoselective adsorbent compared with the blank adsorbent.

Further work has shown that stereoselective adsorbents may be prepared to distinguish between enantiomorphic analgesic type compounds, for example, a gel with footprints of levorphan (II) will adsorb levorphan better than its enantiomorph dextrorphan; the gel will adsorb both enantimorphs better than a blank gel prepared devoid of footprints. Morphine (I), the analgesically active isomer, is adsorbed on a gel with levorphan



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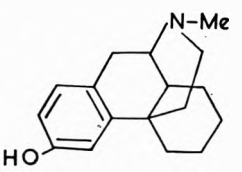
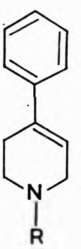


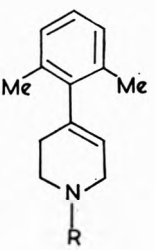
(II)

(analgesically active) footprints slightly better than on a gel with dextrorphan (analgesically inactive) footprints to indicate that probably morphine and levorphan have the same steric arrangement.

Molecular models indicate that the introduction of methyl groups into (III) to yield (IV) will restrict the rotation of the phenyl group so that the aryl group in (IV) will be held approximately at right angles to the general

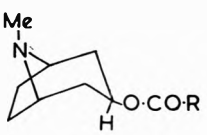


(III)

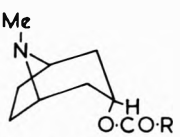


(IV)

plane of the tetrahydropyridine ring. Ultra-violet absorption measurements support this contention since the aryl-double bond conjugation exhibited by (III) is completely absent in (IV). As expected, planar compounds (III) are more readily adsorbed on silica gel than Type (IV) molecules. Footprints on silica of planar molecules (III) show even a greater selectivity for similar planar molecules than for out of plane molecules (IV) than do blank gels.



(V)



(VI)

Investigation of atropine-type molecules indicates that suitable stereoselective adsorbents may be prepared, for example, a gel with footprints of cinnamyltropine (V; R=$C_6H_5CH{=}CH{\cdot}$), adsorbs this compound more readily than cinnamyl-ψ-tropine (VI; R=$C_6H_5CH{=}CH{-}$).

REFERENCE

1. Beckett and Anderson, *Nature, Lond.*, 1957, **179**, 1074.

After Dr. Beckett presented the communication there was a DISCUSSION. The following points were made.

Adsorbents other than silica gel had been tried initially with little success. Preparation of "footprints" in the gels was always good where rigid molecules were used. Flexible molecules did not give such satisfactory results. The molecules trapped in the gels could not be leached out in solution as they were associated with the gels by hydrogen bonding. The exposed surfaces of physically held crystalloids could still be a possible explanation for receptive surfaces. When two isomers were used in preparing the gel two types of "footprint" were formed. Reference molecules must be very stable, as there could be a build up of protons at the gel surface giving acid conditions.

A CONTRIBUTION TO POWDER COMPACTION THEORY BY THE PRESSING OF REGULAR ARRANGEMENTS OF SPHERES

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Received April 30, 1959

Regular arrangements of annealed phosphor bronze spheres have been used to investigate the lubricating effect of graphite films located in various parts of the system being compressed in a cylindrical die. The relative importance of interparticulate lubrication and of die wall lubrication are discussed. The influence of the "angle of packing" on the pressing behaviour has been investigated using similar arrangements of spheres but varying the diameter of the upper layer. From consideration of these experiments, it would appear that die wall friction calculations for powders should include a factor for particle deformation and interparticulate frictional effects.

The elucidation of mechanisms governing the compaction of powders in a die has been hampered by the practical difficulty of isolating a particular effect for detailed study. Some of the problems which require investigation are associated with the friction both at the die wall and between particles. It is of great practical interest to be able to assess whether the use of lubricants is advantageous or otherwise in various parts of a mass being compacted. Some general work has been reported[1–12]. Munzel

TABLE I

Angle of packing and ratio of die wall to applied thrust for various ball systems

Lower ball diameter 0·794 cm.

Upper ball diameter d {in. / cm.}	1/8 0·318	5/32 0·397	3/16 0·476	7/32 0·556	1/4 0·635	9/32 0·714	5/16 0·794
Angle of packing α degrees ..	55·7	50·4	46·2	42·8	39·3	37·5	35·3
tan α	1·465	1·220	1·044	0·925	0·836	0·765	0·708
F_d/F_a for linear portion of curves in Figures 5–7	—	0·124	0·094	0·096	0·084	0·068	0·064

with Kagi[13] and Seth[14] have classified substances used as lubricants for pharmaceutical tabletting into anti-frictional materials, acting at the die wall, and "glidants" which enhance the flow properties of a powder or granulation.

We have described experiments[12] on the compression of cylinders of various materials and single phosphor bronze spheres in an apparatus where the die can be moved relative to the material under compression whilst maintaining and measuring the axial forces exerted by the punches and by the die wall. This work suggested that, within the limit of the applied pressure used, the coefficient of friction at the die wall in a powder

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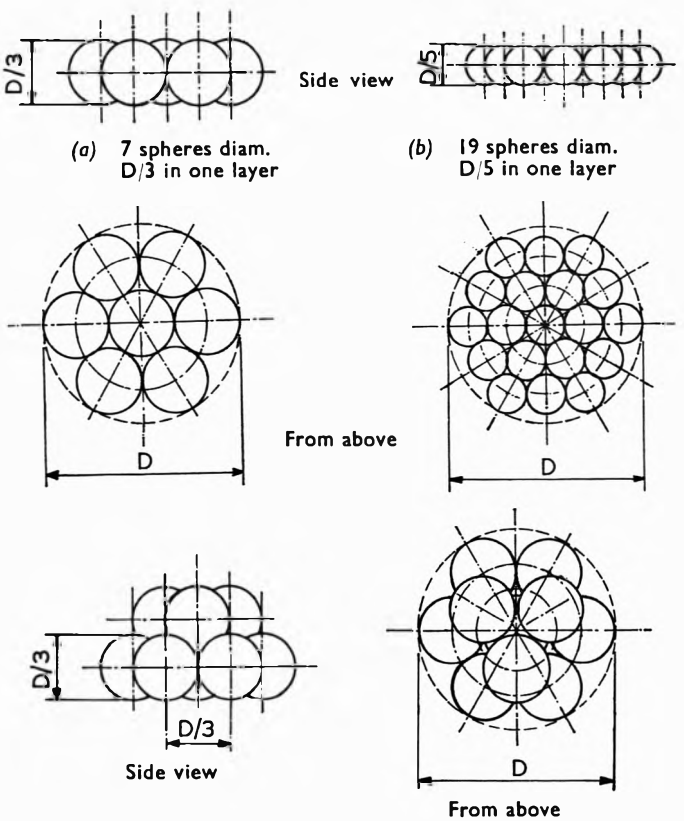

FIG. 1. Arrangement of spheres in a die, bore D.

compact may be constant. It is also nearly independent of the amount of movement, when this is produced at an approximately constant rate, at the interface between the compact and the die wall. But the work did not indicate the effects caused by inter-particulate movement. The experiments now described were made to investigate these effects.

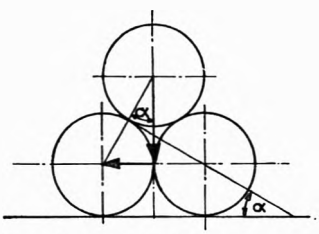

FIG. 2. Angle of packing = α.

Theoretical Considerations

A simplification of the complex physical condition produced by random inter-particulate movement during compression may be made by using a system of spheres. The arrangements used are shown diagrammatically in Figure 1, *a* and *b* for single layers and *c* for a double layer.

With two layer systems there is an initial angle, α, between the horizontal and tangential planes of contact of the two balls in different layers (Fig. 2).

This angle is referred to below as the "angle of packing". Several workers[7,15,16] have assumed that the initial "angle of packing" of a powder is identical with the "angle of repose" of a heap of the same powder, but it has been shown[17] that an experimental value for the angle of repose may

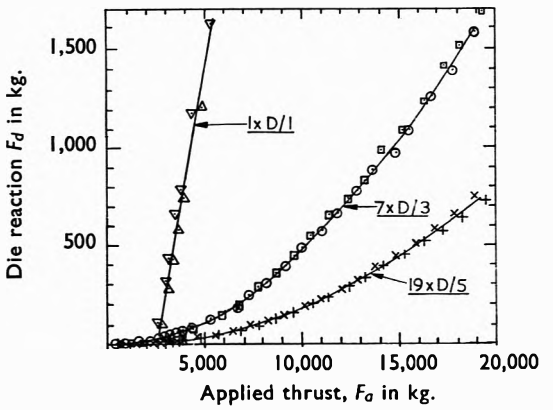

FIG. 3. Pressing single layer arrangements of unlubricated spheres in an unlubricated die of bore 2·38 cm.

△ Exp. I. ▽ Exp. II. System: 1 sphere diam. D/1.
⊙ Exp. III. ⊡ Exp. IV. System: 7 spheres diam. D/3.
+ Exp. V. × Exp. VI. System: 19 spheres diam. D/5.

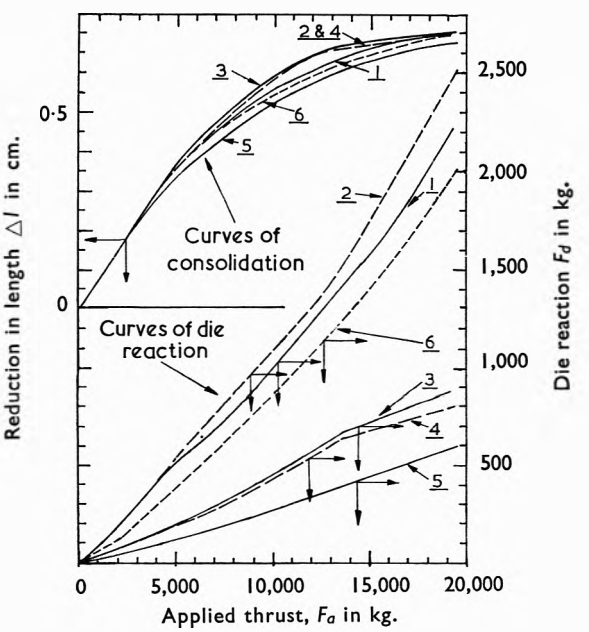

FIG. 4. Pressing double layer arrangements of spheres in a die of bore 2·38 cm. Graphite film on: 1, Punch surfaces. 2, Top layer. 3, All surfaces. 4, Bottom layer and die wall. 5, Die wall. 6, No graphite.

vary markedly with the method of measurement and that data based on angle of repose measurements must be used with caution. For the two layer arrangement shown in Figure 1*c*, it can be shown[18] that α may be varied by altering the diameter, *d*, of the three spheres in the upper layer (Table I).

TABLE II

SUMMARY OF LUBRICATION EFFECTS IN SIMPLE TWO LAYER ARRANGEMENT OF SPHERES

Curve No. on Figure 4	Location of graphite layer	Interface lubricated	Remarks
1	Punch surfaces	Punch surfaces in contact with balls	Die wall friction, F_d, was larger than for the unlubricated pressing. It would be expected that radial movement of the balls would be easier in this instance.
2	Upper layer of balls	Upper punch surface and internal contacts of balls	F_d larger than curve (1) particularly when the applied thrust, F_a, exceeded 5,000 kg., indicating that the effect of internal lubrication was significant above this level of thrust.
3	Both layers of balls, die wall and punch surfaces	Upper and lower punch surfaces, die wall and internal contacts of balls	F_d approximately half that observed for the unlubricated pressing, indicating that die wall lubrication has a sufficiently large influence to counteract punch surface and internal lubrication.
4	Lower layer of balls and die wall	Lower punch surface, die wall and internal contacts of balls	F_d smaller than when all interfaces were lubricated (curve 3), showing that the lubrication of the upper punch surface aids the force transmission in this system.
5	Die wall	Die wall in contact with balls	F_d smallest of all the experiments, since the punch surface and internal friction were high, favouring low radial force transmission, yet coefficient of die wall friction was low.

If interparticulate friction were absent in this system, the ratio of radial force, F_x to the axial force, F_a for practical values of *d* is

$$F_x/F_a = 2/3 \tan \alpha.\cos 30° \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

It might be anticipated that the angle, α, would change during the pressing and that there would be some interparticulate friction. If the factor β is inserted to account for these phenomena,

$$\text{then, } F_x/F_a = 2/3\beta.\tan \alpha.\cos 30° \quad .. \quad .. \quad .. \quad .. \quad (2)$$

but if F_d is the reaction at the die wall, then $F_d/F_x = \mu$, the coefficient of friction between the material and the wall,

$$\text{therefore } F_d/F_a = (2/3\mu.\beta \tan \alpha.\cos 30°) \quad .. \quad .. \quad .. \quad (3)$$

Symbols

D Die diameter.
d Diameter of upper layer of spheres.
F_a Applied axial thrust (or load).
F_d Die reaction in an axial direction.
F_x Radial thrust.
Δl Axial contraction of pressing due to applied thrust.
α Angle of packing.
β Factor connected with the internal characteristics of a multiparticulate system.
μ Coefficient of friction.

Experimental

All experiments were made using phosphor bronze balls of suitable diameter annealed, by heating to 600° for one hour, cooled and polished.

As a preliminary, balls in a single layer were pressed and the die reaction, F_d, was noted for different levels of applied thrust, F_a, of the top punch. The results are given in Figure 3.

Before varying the angle of packing, the effect was investigated of the location of a graphite layer on the packing system of seven D/3 spheres

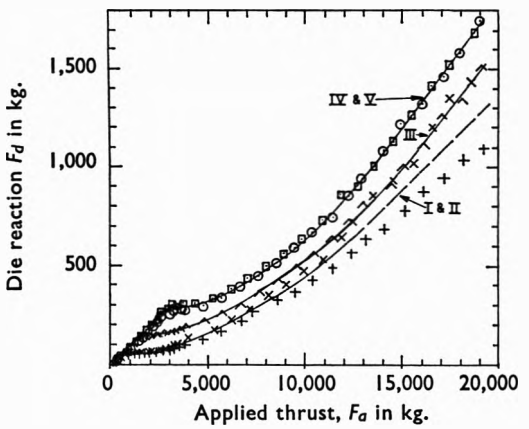

FIG. 5. 3 spheres diameter d, on 7 spheres diameter D/3 in a die diam. 2·38 cm.
+ Exp. I d = 0·3175 cm. × Exp. II d = 0·3175 cm. ∧ Exp. III d = 0·3969 cm.
⊙ Exp. IV d = 0·4763 cm. ⊡ Exp. V d = 0·4763 cm.

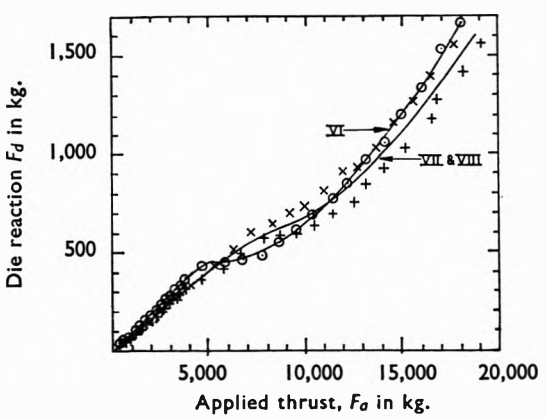

FIG. 6. 3 spheres diam. d on 7 spheres diam. D/3 (cont. from Fig. 5). ⊙ Exp. VI d = 0·5562 cm. + Exp. VII d = 0·6350 cm. × Exp. VIII d = 0·6350 cm.

surmounted by three D/3 spheres in the 2·38 cm. bore die (Fig. 1*c*). The experimental variation of die reaction, F_d, and the contraction, Δl, in compact length with applied thrust, F_a, is shown in Figure 4, whilst a qualitative survey of the effect of the location of the lubricant film is given in Table II.

The effect of variation of the "angle of packing", α, on the pressing-behaviour of the arrangement of spheres shown in Figure 1*c* was investigated by altering the diameter of the balls in the upper layer as indicated in Table I. The systems thus obtained were pressed as before, the balls and the die wall being polished and degreased, and without lubricant in any part of the system. Figures 5–7 show the experimental results.

Discussion

Single layer systems. It will be noted (Fig. 3) that the die reaction with a single ball is greater than with the other arrangements. Subsequent inspection of the compacts confirmed that the area of the zone of contact of the material pressed against the die wall was approximately proportional

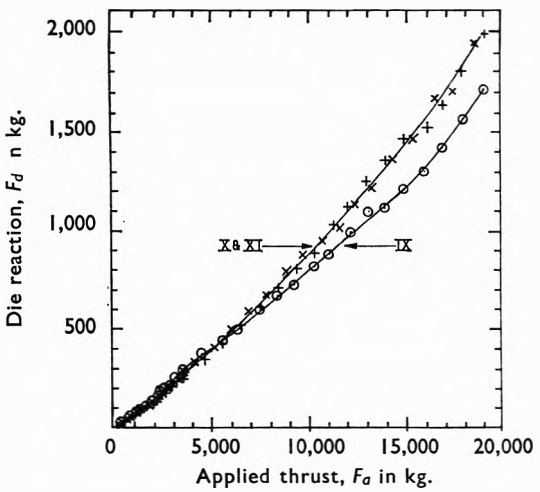

Fig. 7. 3 spheres, diameter d on 7 spheres, diameter D/3 (cont. from Fig. 6). D/3 = 0·7938 cm.
⊙ Exp. IX d = 0·7144 cm. + Exp. X d = 0·7938 cm. × Exp. XI d = 0·7938 cm.

to the magnitude of the die reaction. But it was realised that both size-effects and work-hardening properties of the material were modifying the reactions. The curves in Figure 3 can be considered as showing the order of magnitude of the die reaction and not a direct comparison between different arrangements of balls in a single layer.

Location of lubricant. The changing slopes of the curves (Fig. 4) indicate changes in the mode of force transmission through the system as F_a is increased. The magnitude of the die reaction depends upon two main factors, namely the internal-force-transmission-characteristics and the contact conditions at the interface between the balls and the die wall. Thus die reaction, F_d, is lowest in Curve 5, when there is a high coefficient of interparticulate friction and a low coefficient of die wall friction.

Examination of the curves of reduction in compact length, Δl, against applied thrust, F_a, in Figure 4, shows that consolidation was considerably increased in the mid-stage of the pressing by interparticulate lubrication

and to a lesser extent by lubrication of the punch-compact interface. In both a marked flattening of the $\Delta l/F_a$ curve occurred at higher applied thrust levels, but when the maximum experimental applied thrust was reached, lubricated and unlubricated systems were all consolidated to the same amount $\pm$ 0·25 mm.

These experiments support the suggestion[15] that the effect on die wall friction of a change in the interparticulate coefficient of friction is small compared with changes in the coefficient of friction at the die-wall itself. An effect of the lubricant on the consolidation of the system was evident only at certain stages of the compaction process. This would account for the conflicting evidence on this point which has been reported in the literature[5–8].

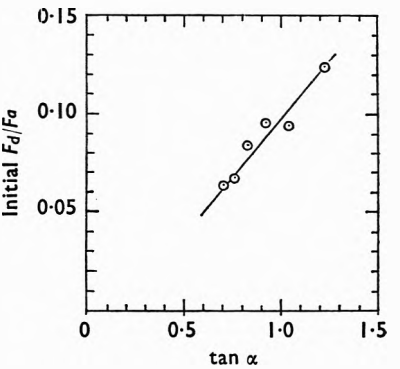

FIG. 8. Variation of F_d/F_a with tan α (please refer to text).

Double layer systems (*Figs.* 5–7). The effect of the diameter of the top layer is well marked, but the same general form of curve may be traced through all the pressings. The "angle of packing", α, would be expected to have the greatest effect on the die-wall friction at the beginning of the pressing. The slopes of the initial linear sections of the curves of die reaction, F_d, against applied thrust, F_a, shown in Figures 5–7 are recorded in Table I.

Figure 8 shows that, in this section, F_d/F_a equals 0·12 tan α and, substituting in equation (3), $2/3\,\mu.\beta \cos 30° = 0{\cdot}12$.

If the coefficient of friction, μ, is constant[12], it may therefore be stated that the factor, β, in the first stage of the pressing, is independent of the "angle of packing", α. It is likely that over a given range of applied stress, β depends upon those properties of the material that also control the so-called coefficient of interparticulate friction.

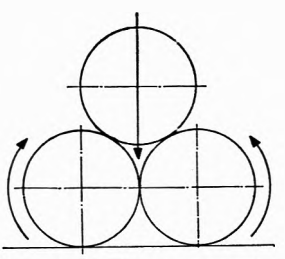

FIG. 9. Effect of interparticulate movement. Top ball moving downwards causing tendency for rolling in lower layer.

This factor, β, has been ignored in the past, and such an omission may account for the low value of the coefficient of friction, μ, at the die wall, calculated when pressing unlubricated copper powder[16].

At certain stages in some pressings, the die moved upwards, that is, in the opposite direction to the applied thrust, as the applied thrust F_a, was increased. After ejection of the pressing, marks on the balls in the lower layer suggested that each ball had been rotated owing to the insertion of the upper layer into the interstitial spaces of the lower layer, as shown in Figure 9. This action appeared to be governed by the ease with which the upper layer could be

forced into the spaces and by the relationship between interparticulate and die wall friction.

A similar effect has previously been noted[8] when pressing powders. This phenomenon may be of importance in powder compaction and would bear further investigation.

Acknowledgements. We are grateful to Mr. J. J. Deer, School of Pharmacy, for the precision machining.

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REFERENCES

1. Sheinhartz, McCullough, and Zambrow, *J. Metals, N.Y.*, 1954, **6**, 515.
2. Spencer, Gilmore, and Wiley, *J. appl. Phys.*, 1950, **21**, 527.
3. Taylor, *Trans. Inst. min. Engrs.*, 1938, **96**, 153.
4. Toor and Eagleton, *Industr. Engng Chem.*, 1956, **48**, 1825.
5. Seelig and Wulff, *Trans. Amer. Inst. min. (metall) Engrs.*, 1946, **166**, 492.
6. Bal'shin, *Vestnik Metalloprom*, 1938, **18**, 124.
7. Torkar, *Arch. Eisenhüttenw.*, 1956, **27**, 285.
8. Ballhausen, *Lecture at the Powder Metallurgy Conference, Aachen, Germany*, 27th June, 1957.
9. Shank and Wulff, *Trans. Amer. Inst. min. (metall.) Engrs.*, 1949, **185**, 561.
10. Duwez, and Zwell, *ibid.*, 1949, **185**, 137.
11. Nelson, *J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 494.
12. Train and Carrington, *in the press.*
13. Munzel and Kagi, *Pharm. Acta. Helvet.*, 1957, **32**, 321.
14. Seth, Sc.D. Thesis, Zurich, 1956.
15. Ballhausen, C., *Arch. Eisenhüttenw.*, 1951, **22**, 185.
16. Duffield and Grootenhuis, *Iron and Steel Inst. Spec. Rpt.*, 58, 1954.
17. Train, *J. Pharm. Pharmacol.*, 1958, **10**, Suppl. *127T.*
18. Carrington, Ph.D. Thesis, London, 1958.

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

PHARMACOLOGICAL AND CHEMICAL OBSERVATIONS ON SOME TOXIC NECTARS

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Received April 20, 1959

Acetylandromedol (andromedotoxin) has been isolated from the nectar of *Rhododendron thomsonii*. The pharmacological properties of toxic nectars obtained from several *Rhododendron* sp. and hybrids have been investigated and these indicate that the poisonous principle is acetylandromedol.

Bees which consume nectars containing toxic substances may produce poisonous honey. Reports of poisoning of bees by nectars from plants of the *Ericaceae* are not numerous and reports of systematic chemical and pharmacological investigations of the nectars themselves have not appeared in the literature. Honey found near Trebizonde in northern Asia Minor poisoned Xenophon's troops[1] and Pliny[2] describes the poisonous honey found at Heraclea in the Pontus in Asia Minor as having a strong odour causing sneezing and if eaten, thirst, perspiration and pain. Strabo,[3] a native of the Pontus, mentions the intoxication of three of Pompey's armies by honey eaten as they were crossing the mountains of Themiscyra in Asia Minor and their consequent destruction by a local tribe. Thresh[4], Krause[5], Pulewka[6] and Ungan[7] have given more recent descriptions of the history and properties of poisonous honey.

Nectars taken from several families are believed to produce poisonous honey but most of it comes from the *Ericaceae*. *Rhododendron*, *Azalea*, and *Andromeda* sp. are especially important. Plants from these species contain the complex, non-nitrogenous compound andromedotoxin (Plugge[8–10]) isolated from Trebizonde honey[4,6]; from *Andromeda japonica* by Eykman[11,12] who called it asebotoxin and from *Rhododendron* sp.[8–10,13,14]. It was also obtained by Horning and his co-workers[15,16] from *R. maximum* and *Kalmia angustifolia* var. *caroliniana* and identified as the acetyl derivative of the glycol, andromedol and hence called acetylandromedol. Rhodotoxin[17–20] and grayanotoxin I[20,21] are identical with andromedotoxin[16,20]. There are reports of a number of plants being poisonous to bees and in some instances *Rhododendron* sp. have been suspect[22,24]. A serious outbreak of poisoning of bees by *Rhododendron* sp. on the island of Colonsay led us to investigate the nature of the toxic substance, to attempt to identify it and the rhododendrons which produced it.

Methods

Nectars were collected from rhododendron flowers (Tables I and II) with a Pasteur pipette and stored in glass stoppered bottles at 4° after removal of pollen grains by centrifugation.

Pharmacology

Bees. For toxicity tests with bees, measured volumes of centrifuged nectars were put into glass vials inverted over holes in the tops of cages. These contained 15 to 25 bees and were kept in a dry incubator at 28°. Control solutions contained 30 per cent w/v of sucrose or 10 and 100 μg./ml. of andromedotoxin in 30 per cent w/v of sucrose. The observation period was 48 hours.

Mice. Groups of from six to eight female mice weighing 30 to 40 g. were given the nectars by intraperitoneal injection. A 1 mg./ml. solution of andromedotoxin was used as control. A few nectars were tested by injecting them intraperitoneally into frogs.

Cat respiration and blood pressure. Cats of either sex weighing between 1·5 and 3·5 kg. were anaesthetised with intraperitoneal sodium pentobarbitone or chloralose. Blood pressure was recorded from the common carotid artery and injections made into the cannulated external jugular vein. Respiration was recorded directly from the movements of the epigastrium by means of a thread attached to a lever, or from the movements of a tambour connected to a cannula inserted into the trachea.

Other experiments. Doses of up to 1 ml. of nectar were injected into the cannula supporting the isolated cat heart perfused by Langendorff's method[26]. The effects were compared with those after injection of 0·1 to 0·5 mg. of andromedotoxin. In a few experiments the effects were investigated of intravenous injection of up to 100 μg./kg. of andromedotoxin and 0·5 ml. of nectar upon the twitch height of the cat gastrocnemius muscle stimulated indirectly *via* the sciatic nerve. The direct action of nectars on isolated strips of guinea pig ileum suspended in Tyrode's solution at 36° and on acetylcholine-induced contractions of the frog rectus muscle suspended in frog Ringer's solution at room temperature were also investigated.

Results

Toxic nectars (Tables I and II) produced the following effects. Bees became dull, sluggish or inert, flew only in short spurts, falling from time to time to the floor of the cage. They then lay on their sides or backs, the abdomen turned upwards as if the bee was supported by its wings. There was ataxia and disorientation, bees climbing downwards not upwards as is usual. When taken out of the cage the bees spun round characteristically and vibrated their wings very rapidly but could not fly. There was increasing weakness and prostration and death followed, the tongue often being extended.

Mice became quiet, the flanks drawn in and there was a tendency to drag the hind limbs and abdomen along the ground. Sharp spasmodic contractions of the diaphragm and gasping movements of the mouth followed. There was salivation, dyspnoea and weakness but when disturbed the animals were still able to move fairly rapidly. Convulsions ensued and after one of these episodes respiration did not return. The heart continued to beat for about one minute after respiration failed.

TABLE I

TOXICITY OF NECTARS FROM CERTAIN *Rhododendron* SPECIES TO MICE AND BEES

Series	Species	Bees		Mice
		No. per group	48 hr. mortality per cent	Approx. LD50 ml./kg.
Arboreum	*R arboreum*	21	62	>20+
,,	*R. arboreum* var. *album*	46	87	>20+
,,	*R. arboreum* var. *hermisinum*	45	16	—
,,	*R. niveum*	27	70	>20++
Barbatum	*R. barbatum*	32	40	—
Falconeri	*R. fictolacteum*	45	0	2·5
Fulvum	*R. fulvum*	23	26	>30++
Grande	*R. sinogrande*	41	44	10
	R. macabeanum	35	14	—
Neriiflorem	*R. sperabile*	32	0	>20++
	R. neriiflorem	107	0	>30++
	R. sperabiloides	32	0	>20++
	R. scyphocalyx	29	0	>20++
	R. euchaites	25	0	>20++
	R. haematodes	25	0	>20++
Taliensi	*R. pratti*	47	85	>20+
Thomsonii	*R. thomsonii*	168	100	10
Andromedotoxin	100 µg./ml.	49	84	1 mg./kg.
,,	10 µg./ml.	51	24	

+ no deaths but toxic symptoms } at highest dose used.
++ no deaths or toxic symptoms }

TABLE II

TOXICITY OF NECTARS FROM CERTAIN *Rhododendron* HYBRIDS TO MICE AND BEES

Name	Parents	Bees		Mice
		No. per group	48 hr. mortality per cent	Approx. LD50 ml./kg.
R. Dicharb.	*R. arboreum* × *R. dicroanthum*	37	0	>20*
R. Red Admiral	*R. arboreum* × *R. thomsonii*	53	59	—
R. Fiery Cross	*R. barbatum* × *R. griffithianum*	25	100	7
R. Abbot	*R. thomsonii* × *R. delavayi*	36	31	720*
R. Barclayi	*R. thomsonii* × R. Glory of Penjerrick (R. Glory of Penjerrick = *R. arboreum* × *R. griffithianum*)	88	97	7
R. Barclayi var. Helen Fox	ditto	84	100	—
R. Red Star	*R. thomsonii* × R. Ascot Brilliant (R. Ascot Brilliant = *R. thomsonii* ×—)	34	100	7
R. Redwing	R. Barclayi (see above) × R. shilsonii (R. shilsonii = *R. thomsonii* × *R. barbatum*)	29	0	>20*
R. J. G. Millais	*R. thomsonii* ×—	27	100	7
—	R. Barclayi × *R. meddianum*	27	100	5
R. May Day	*R. griersonianum* × *R. haematodes*	20	0	—
R. Ascot Brilliant	*R. thomsonii* ×—	19	37	—
—	*R. thomsonii* ×—	26	100	5
—	*R. thomsonii* ×—	19	100	5
Andromedotoxin	100 µg./ml.	49	84	1 mg./kg.
,,	10 µg./ml.	51	24	

* No deaths, but toxic symptons at highest dose used.

In frogs, respiration became gasping, the animals became weak, prostration followed and there were occasional convulsive movements.

Pulewka[6] used mice and frogs to identify poisonous honey. After subcutaneous injection of extracts the animals showed characteristic respiratory disturbances with convulsive movements of the diaphagm, contraction of the bronchial and glottal muscles and movements which simulated vomiting.

When injected into cats, toxic nectars (0·1 to 0·5 ml./kg.) caused depression of respiration associated with contractions of the diaphragm, bradycardia and either a sharp, short-lived fall in blood pressure often followed by a smaller rise, or a sustained hypertensive effect. Similar effects were obtained when 10 to 40 μg./kg. of andromedotoxin were injected. Bradycardia and hypotension were abolished by 1 mg./kg. of atropine sulphate. Cutting the vagi abolished the respiratory and hypotensive effects. The pressor response was abolished by 2 mg./kg. of phentolamine.

Moran and his colleagues[25] similarly showed intravenous injections of low doses of andromedotoxin (2 to 3 μg./kg.) into dogs to cause bradycardia, hypotension and respiratory depression. At higher dose levels (40 to 80 μg./kg.) there was hypertension due to release of adrenaline from the adrenal medullae. Bradycardia was prevented by atropine which also reduced the hypotensive effect. Bradycardia, hypotension and respiratory depression were abolished by vagotomy. Hypertension was prevented by adrenalectomy or phentolamine. Hardikar[13] obtained similar results.

Toxic nectars and andromedotoxin slowed the isolated cat heart and increased the amplitude of the beat. No effect was observed upon twitch height in the gastrocnemius muscle. The nectars (0·01 to 0·03 ml./ml.) caused direct contractions of the isolated guinea pig ileum (Fig. 1) which were inhibited by atropine sulphate. There was no inhibition of acetylcholine-induced contractions of the frog rectus by up to 0·3 ml./ml. of nectar.

Chemical Observations

Isolation and identification of acetylandromedol (andromedotoxin). 240 ml. of nectar of *R. thomsonii* was divided into four 60 ml. portions and each portion simultaneously extracted twenty times in succession with 50 ml. portions of chloroform on a power-driven shaker, allowing five minutes each time for equilibration. Previous experiments had shown that a rise in temperature caused by the use of a continuous chloroform extractor caused extensive decomposition of the chloroform-soluble material. The combined chloroform extracts were concentrated to about 10 ml. at 30° under reduced pressure. The concentrate was treated three times in succession with 20 ml. portions of dry benzene and taken to dryness at room temperature under reduced pressure to ensure removal of all traces of water. A solid residue (42 mg.) was obtained. Fractional crystallisation from chloroform using the Craig tube method[27,28] gave as the least soluble fraction colourless needles

(29 mg. representing 10·8 mg./100 ml. of nectar). The remaining material, which was soluble in light petroleum, was not investigated further. The crystalline solid showed appreciable variations in melting point depending upon the rate of heating but the values always fell within the limits 260 to 272°.

The material showed no absorption in ultra-violet light and was laevo-rotatory. $[\alpha]_D = -8°$ ($c = 1{\cdot}83$ in ethanol); the figure reported for acetylandromedol[15] was $[\alpha]_D = -8{\cdot}8°$ in ethanol. On treatment of the

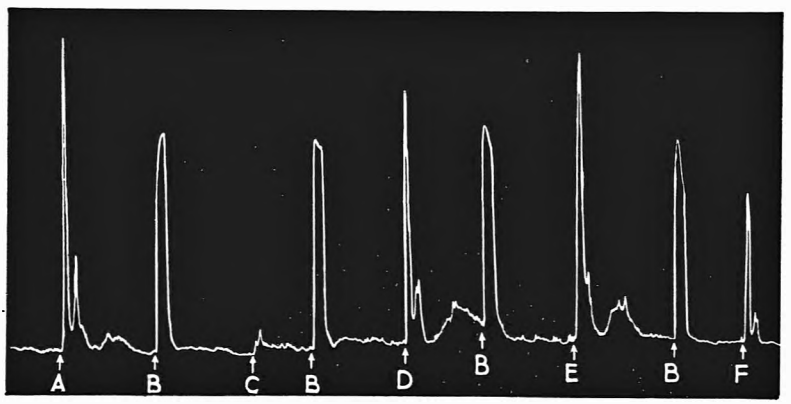

FIG. 1. The effects of rhododendron nectars on the isolated guinea pig ileum 8 ml. bath containing Tyrode's solution at 36°.
At B. 0·3 μg. of acetylcholine chloride.
At E. 30 μg. of acetylandromedol.
At A. 0·3 ml. of nectar from R. Barclayi × *R. meddianum.*
At C. 0·3 ml. " " *R. arboreum.*
At D. 0·3 ml. " " R. Red Star.
At F. 0·3 ml. " " *R. thomsonii.*

solid with mineral acids a deep red colouration was produced and the compound gave a positive reaction with periodic acid. These properties are all in agreement with those reported for acetylandromedol[15]. A direct comparison of the infra-red spectra in KCl discs of our specimen with that of a specimen of authentic acetylandromedol kindly supplied by Dr. E. C. Horning and with the specimen used in the pharmacological tests, confirmed the identity of the three specimens. There was no depression of the melting point on admixture of the compounds.

DISCUSSION

This investigation has shown that some rhododendron nectars are toxic to bees, mice and cats. *R. thomsonii* and some of its hybrids are especially poisonous. *R. arboreum* var. *album* and *R. pratti* are also toxic. The isolation and identification of acetylandromedol (andromedotoxin) from the nectar of *R. thomsonii* and the similar effects seen when toxic nectars and acetylandromedol are tested pharmacologically, indicate that this is the poisonous substance. It is not, however, possible to predict that a particular rhododendron will secrete a toxic nectar: for example,

the hybrid R. Redwing which is derived from four species, three of which are toxic, has been found to secrete a non-toxic nectar (Table II). The significance of these results to bee-keepers will be discussed elsewhere.

Acknowledgements. Permission to collect nectars from their garden on the Isle of Colonsay was kindly given by the Dowager Lady Strathcona and Mount Royal. We thank Mr. and Mrs. J. W. H. Younger of Eckford. near Dunoon, for samples from their garden, Mr. H. N. Davidian of the Royal Botanic Gardens of Edinburgh who identified the rhododendrons, Dr. Glenn Ullyot of Smith, Kline and French Laboratories, Philadelphia, U.S.A., for the sample of andromedotoxin used in the pharmacological studies, Miss Irene Moon and Mr. Charles Dzewu for technical assistance.

References

1. Xenophon, *Anabasis*, Bk. IV, cap. 8, para. 20.
2. Pliny, *Natural History*, Bk. XXI, cap. 44.
3. Strabo, *Geography*, Bk. XII, cap. 3, para. 18.
4. Thresh, *Pharm. J.*, 1887, **18**, 397; 404.
5. Krause, *Naturwissenschaften*, 1929, **44**, 976.
6. Pulewka, *Rev. fac. Med. Univ. Istanbul*, 1949, **12**, 275.
7. Ungan, *Turk. Z. Hyg. exp. Biol.*, 194, **2**, 166.
8. Plugge, *Arch. Pharm. Berl.*, 1883, **221**, 1; 813.
9. Plugge, *ibid.*, 1885, **223**, 905; 914.
10. Plugge, *ibid.*, 1891, **229**, 554.
11. Eykman, *Rec. Trav. chim. Pays-Bas*, 1882, **1**, 224.
12. Eykman, *Ber. Dtsch. chem. Ges.*, 1884, **16**, 2769.
13. Hardikar, *J. Pharmacol.*, 1922, **20**, 17.
14. Archangelsky, *Arch. exp. Path. Pharmak.*, 1901, **46**, 313.
15. Wood, Stromberg, Keresztesy and Horning, *J. Amer. chem. Soc.*, 1954, **76**, 5689.
16. Tallent, Riethof and Horning, *ibid.*, 1957, **79**, 4548.
17. Makino, *Okayama Igakkai Zasshi*, 1927, **39**, 2099.
18. Makino, *ibid.*, 1928, **40**, 138.
19. Kohanawa, *Jap. J. Pharmacol.*, 1956, **6**, 46.
20. Takemoto, Nishimoto, Meguri and Katayama, *J. pharm. Soc. Japan*, 1955, **75**, 1441.
21. Takemoto, Nishimoto, Meguri and Katayama, *ibid.*, 1958, **78**, 110.
22. Maurizio, *Schweizerischen Bienen-Zeitung*, 1945, Supp. 9, 430.
23. Howes, *Plants and Bee Keeping*, Faber and Faber, London, 1945.
24. Grout, *The Hive and the Honey Bee*, Dadent and Sons, Hamilton, Illinois, 1949.
25. Moran, Dresel, Perkins and Richardson, *J. Pharmacol.*, 1954, **110**, 415.
26. Gunn, *J. Physiol.*, 1913, **46**, 506.
27. Craig and Post, *J. industr. Engng Chem.* (*Anal. Ed.*), 1944, **16**, 413.
28. Martin-Smith, *Lab. Pract.*, 1958, **7**, 572.

After Miss Carey presented the paper there was a DISCUSSION.

Short Communication

INDOLE DERIVATIVES IN TOMATOES

By G. B. West

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Received May 2, 1959

During systematic examination of edible fruits, the banana[1] and the tomato[2,3] have been found to contain indole derivatives. Whereas the banana contained much 5-hydroxytryptamine (5-HT), the predominant indole derivative in the tomato was shown to be tryptamine. This communication describes the initial work being carried out to trace the formation and assess the importance of these indole compounds in the life history of the tomato plant.

As soon as the first flowers had formed on young tomato plants the different parts were dissected for extraction. The tomato fruit was collected at different stages of maturation, from green and about 1 in. in diameter till over-ripe and very soft. Each of these fruits was then dissected into the skin, the pulp and the pips. Extracts were made with acetone (1 g./5 ml.) for 24 hours. After reducing the extracts to a small volume, aliquots were either tested for 5-HT activity on the isolated uterus of the rat in oestrus or subjected to two-dimensional paper chromatography. The solvents were sodium chloride solution (8 per cent w/v), isopropanol : ammonia : water (20 : 1 : 2), and *n*-butanol : acetic acid : water (4 : 1 : 5). The indoles were detected on the chromatograms with Ehrlich's reagent. Duplicate spots were eluted and the eluates tested biologically for 5-HT activity. The concentrations of other indole derivatives were estimated visually by comparison with the colours produced by known amounts of standard substances. These were 5-HT creatinine sulphate, tryptamine hydrochloride (T), tryptophan (TP), 5-hydroxytryptophan (5-HTP), 5-hydroxyindoleacetic acid (5-HIAA), and indoleacetic acid (IAA). All values shown are the means of three observations.

Young tomato plants. No indole derivatives were detected in extracts of the roots of these plants. In the main stem, TP was found in a concentration of 2·5 μg./g. The concentration of 5-HT in this region was less than 0·01 μg./g. When the leaf stems were examined, the concentration of TP had not increased but that of 5-HT was 0·3 μg./g. In the leaves and leaflets 5-HT was present in an even higher concentration (0·5 μg./g.). Lastly, in the flowering tip, both TP (2·5 μg./g.) and 5-HT (0·3 μg./g.) were detected. These results are shown in Table I. T, 5-HTP, 5-HIAA and IAA were not detected in any of the extracts obtained from the parts of the young tomato plants.

Tomato fruit. When the green tomato fruit was examined, the predominant indole was T (1 μg./g.), and smaller amounts (0·2 μg./g.) of both 5-HT and TP were also present. In the ripe fruit, the concentration of T

always exceeded that of 5-HT, the highest ratio of the two levels being in the pips (see Table I). When washed free from pulp, the pips on extraction were found to possess more than nine times as much T and 5-HT. The concentrations of TP in these extracts were relatively small. Ripe tomatoes secured from the Canary Islands gave results which were similar to those described above for the variety obtained from the Channel Islands. The results with extracts of over-ripe fruits indicated a slight loss in the 5-HT activity and in the T content. Tinned Italian tomatoes also contained 5-HT and T but the concentrations of each varied widely.

TABLE I

ESTIMATES OF 5-HT AND T (μg./g.) IN PARTS OF THE TOMATO PLANT AND FRUIT

Indole compound	Young plant			Fruit			
	Main stems	Leaf stems	Leaves	Unripe pulp	Ripe skin	Ripe pulp	Ripe pips
5-HT	0	0·3	0·5	0·2	1·5	3·4	1·0
T	0	0	0	1·0	1·8	4·0	4·8
TP	2·5	2·5	2·0	0·2	0·2	0·8	0·4

The high concentration of T, an indole compound, in the fruit of the tomato, and especially in the pips of the ripe specimens examined, suggests that it may play a role in metabolism such as regulating or stimulating new growth. However, no T has so far been detected in extracts of the young plant so it is probable that one of the other indole compounds performs this function at this stage of growth. On the other hand, T may be a precursor of 5-HT which itself may be the important stimulator of growth in the tomato. It is of particular interest that 5-HTP was not detected as this amino acid is generally recognised as an intermediate in the formation of 5-HT in animals, and T is not often found in the animal kingdom. It is probable that 5-HT in the tomato is formed from the stores of TP.

A suggestion that 5-HT is possibly the counterpart in animals of auxin (3-indoleacetic acid, IAA) in plants was put forward by Woolley[4] in 1957. Auxin causes plant cells to grow, most probably as it increases the uptake of water. 5-HT also alters the permeability of the animal cell wall. Experiments by Pickles and Sutcliffe[5] further showed that both auxin and 5-HT changed the permeability of the cells in slices of beetroot so that the red pigment was released into the surrounding medium. As no IAA has so far been detected in the tomato extracts, it may be that 5-HT which is present in the young plant as well as in the fruit is one of the hormones of growth in the tomato. 5-HT was not found in the main stems of the young plant, but this part of the plant is where transport (and not necessarily growth) is the essential process. Further work is now needed to determine at what stage in the life history of the plant the changeover from TP to T occurs and the role of T in the development of the fruit.

This work represents a fresh approach to the significance of 5-HT in nature. It may be possible to determine the type of cell producing 5-HT and so reach a stage nearer to finding its function in biology.

REFERENCES

1. Walkes, Sjoerdsma, Creveling, Weissbach and Udenfriend, *Science*, 1958, **127**, 648.
2. West, *J. Pharm. Pharmacol.*, 1958, **10**, 589.
3. West, *ibid.*, 1959, **11**, 319.
4. Woolley, *Nature, Lond.*, 1957, **180**, 630.
5. Pickles and Sutcliffe, *Biochem. et Biophys. Acta*, 1955, **17**, 244.

After the Author presented the communication there was a DISCUSSION.

THE CHARACTERISATION OF CRYSTALLINE AND AMORPHOUS ALOIN

By R. E. Lister and R. R. A. Pride

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Received May 29, 1959

Tests are described showing the differences between amorphous aloin and that of the B.P. 1953. One of these tests might, with advantage, be incorporated in official publications to differentiate crystalline aloin from the relatively impure and less active amorphous aloin.

An amorphous aloin which does not conform to the description in the British Pharmacopoeia, 1953, has appeared in commerce, particularly in the export market. Aloin is described as "a pale yellow, microcrystalline powder"; the amorphous material shows no trace of crystallinity under the polarising microscope. Other samples appeared to contain a mixture of amorphous and crystalline material.

Crystalline aloin of the Pharmacopoeia is obtained mainly from Cape and Curaçao aloes by a process involving precipitation of the calcium salt, subsequently decomposed by hydrochloric acid. The product consists of small yellow crystals, soluble in about 100 parts of water which contain a high proportion of barbaloin[1,2]; the yield is about 12 per cent from Cape and 25 per cent from Curaçao aloes.

Amorphous aloin is obtained in about 60 per cent yield from a dried extract of Cape aloes, free from resinous material, by solvent extraction. The sample thus prepared was compared with a sample of German origin. Both were yellow powders, low in barbaloin content[1] and very soluble in water; both turned rapidly to a sticky paste with a few drops of water. This simple test will distinguish amorphous aloin from that conforming to the B.P., 1953, standard, which remains mostly solid when a small quantity of water is added. The two amorphous samples were identical, as shown by ultra-violet spectrophotometry and paper chromatography.

The catharsis produced by a German sample of amorphous aloin was investigated by Auterhoff and Ball[2]. Tests on human subjects indicated an effective dose for amorphous aloin more than double that for crystalline aloin, and about two thirds that for Cape aloes. A satisfactory method of bioassay for aloes extracts using rats was described[3], and this was used by us.

Experimental

The following materials were used in the experiments: Crystalline aloin (B.P. 1953), amorphous aloin, amorphous aloin (German), barbaloin, purified as described by Hay and Haynes[4], and Cape aloes, powdered.

Ultra-violet Absorption Spectra

The curves (Fig. 1) were obtained with aqueous solutions, concentration 0·0025 per cent w/v, in 1-cm. quartz cells on a Unicam S.P. 500 spectrophotometer. The spectra of the amorphous samples were identical, with maxima at 254 and 298 mμ [E (1 per cent, 1 cm.) values of about 300 and 320, respectively]. The spectrum of aloin, B.P., resembled that of barbaloin, differing only slightly in intensity at the maxima at 269, 298 and 354 mμ [E (1 per cent, 1 cm.) values 190, 225 and 245 for aloin and 215, 240 and 260 for barbaloin, respectively]. The spectrum of aloin, B.P., thus shows a maximum (at 354 mμ) absent from that of the amorphous material. Measurement of the relative intensities at 354 and 298 mμ provides a convenient means for distinguishing crystalline aloin from amorphous.

Absorption spectra previously reported for crystalline aloin[1,5] and for German amorphous aloin[1] were similar to those of Figure 1.

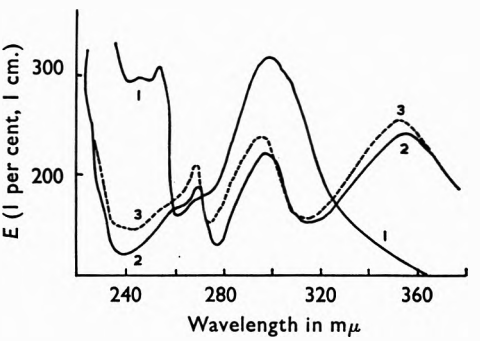

FIG. 1. Ultra-violet absorption spectra. 1. Amorphous aloins. 2. Aloin B.P. 3. Barbaloin.

Paper Chromatography

Several workers have investigated the separation of aloes constituents by paper chromatography; most of them favoured a solvent mixture comprising *n*-butanol, acetic acid and water[1,6–8]. This mixture appeared to have no advantage over *n*-butanol saturated with water which we used. Spots of aqueous solutions, of the following concentrations, were applied at the starting line on sheets of Whatman No. 2 paper. Amorphous aloin (German), 0·4 per cent w/v; barbaloin, 0·2 per cent w/v; amorphous aloin, 0·4 per cent w/v; aloin, B.P., 0·25 per cent w/v, and Cape aloes, 2·0 per cent w/v.

Development was by the ascending method for 30 hours at 20° $\pm$ 1·5. When the paper was dried, and examined under ultra-violet illumination from a Hanovia "Chromatolite", the spots were seen to be clearly separated. Every sample on the paper showed a spot with dark orange fluorescence changing to bright yellow in contact with ammonia vapour. This spot proved to be barbaloin (R_F about 0·77). Crystalline aloin (B.P. 1953) showed the barbaloin spot only. In both the samples of amorphous aloin and in the sample of Cape aloes, the yellow barbaloin spot was between two bright blue fluorescent spots (R_F values about 0·57 and 0·87). Similar spots observed by previous authors were reported as *p*-cumaric acid and an unknown anthracene derivative[8].

The chromatograms were also photographed on reflex-copying paper by an ultra-violet printing technique similar to that of Markham for nucleic acids[9]. Negatives obtained in this way were re-printed as positives

in a photo-copier. The enhanced contrast of the final prints rendered the spots clearly visible. The two amorphous aloin samples were seen to be alike, and to have the same principal constituents as the water-soluble fraction of Cape aloes.

Assay of Aloin

No satisfactory chemical method of assay is known for aloes preparations (see, however, ref. 1). Consignments of aloes are usually assessed by the isolation of aloin. Typical recoveries of aloin from Cape aloes are 12 to 15 per cent. The procedure was applied to amorphous aloin and to aloin, B.P., as a further means of distinction; the yields obtained were markedly different.

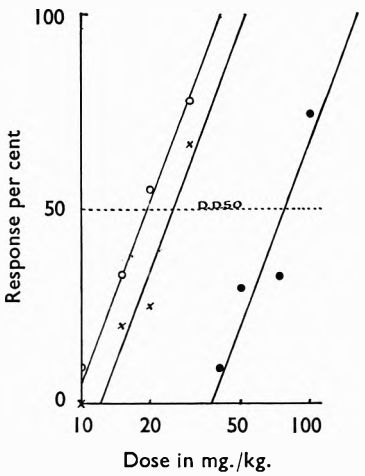

FIG. 2. Dose response curves for three samples of aloin. ○—○, Barbaloin. ×—× Aloin, B.P. 1953. ●—●, Amorphous aloin.

Amorphous aloin (5 g.) was dissolved in 350 ml. of warm, dilute hydrochloric acid (pH about 3·0). The insoluble residue was filtered and washed with a further 50-ml. portion of the same acid. Filtrate and washings were combined and cooled to room temperature. Calcium hydroxide (1·25 g.) was added and the mixture well stirred. Hydrochloric acid was added until the solution was just moderately alkaline, and the mixture was stirred for 90 minutes. The precipitate was then filtered in a Buchner funnel, sucked dry, and washed with two 10-ml. portions of ice-water. It was then transferred (as rapidly as possible) to a small beaker where it was mixed with concentrated hydrochloric acid (2·5 ml.). The mixture was gently warmed until a clear solution was obtained. This was left overnight in the refrigerator. The product was filtered in a sinter-glass crucible, sucked dry, and washed with three 2-ml. portions of ice-water. It was then dried to constant weight in an oven at about 60°. The yield of crystalline aloin was 16 per cent, this low value being explicable in terms of the solubility of the calcium aloin. In a similar experiment with aloin, B.P., the yield was 91 per cent.

Bioassay

Barbaloin, aloin, B.P., and amorphous aloin were assayed by the method of Latven and others[3] for aloes extracts. This is based on an assessment of the consistency of rat faecal pellets. Oral administration of aloin to rats produces catharsis characterised by light-coloured, soft, wet faecal pellets. This catharsis reaches a maximum at about 12 hours and persists for a further 12 hours; for convenience the assessment was made 17 hours after the animals were dosed.

The faecal consistency was assessed by allowing a faecal pellet to fall on to a clean glass plate; a hypodermic needle of 0·9 mm. diameter was inserted vertically downwards into the pellet and lifted. If the pellet adhered to the plate it was scored positive, if it was lifted by the needle (as was a normal pellet from an untreated animal) it was scored negative. Four groups of four female rats in the weight range 140 to 190 g. were used to test each sample.

The median defaecatory dose (DD50) for each sample was determined as described in the original paper[3]. When the percentage response was plotted against log-dose, straight lines were obtained (Fig. 2). These

TABLE I

THE DD50 OF ALOIN PREPARATIONS ADMINISTERED ORALLY TO FEMALE RATS

Preparation	Moisture content percentage w/w	DD50 and limits of error P = 0·95	Relative potency (after correction for moisture content)
Barbaloin	0	19·5 (15·3–25·0)	100
Aloin (B.P. 1953)	6	25·3 (17·3–37·0)	82
Amorphous aloin	8	78·2 (58·9–103·8)	27

lines showed no significant deviation from parallelism, which indicated that the three samples were acting in a similar manner.

The DD50 for each of the three samples, and their relative potencies after correction for moisture content, are given in Table I.

DISCUSSION

Sufficient evidence has been presented to show the marked differences between amorphous aloin and aloin as described in the British Pharmacopoeia, 1953. The first point of difference is, obviously, the amorphous nature of the material. The second is the high water-solubility. The two forms can further be distinguished by differences in the ultra-violet absorption spectra or paper chromatograms. The same criteria emphasize the close similarity of the amorphous material and the corresponding sample of German origin.

It is thus demonstrated that the amorphous type of aloin is a heterogeneous substance, low in barbaloin content, comprising the main water-soluble constituents of Cape aloes. Biological assay has shown that the potency of the amorphous aloin is about one third that of aloin, B.P.

Conclusions. It would be desirable to include a more specific test for crystalline aloin in official publications in view of the appearance in commerce of amorphous material, of different constitution, and lower biological potency. A suitable test involves measurement of the ratios of absorption intensities at 354 and 298 mμ: for crystalline aloin the intensity at 354 mμ is greater than that at 298 mμ; amorphous aloin, however, shows no maximum in the region of 354 mμ, where the intensity is less than that at 298 mμ.

The following description, therefore, specifies crystalline aloin:

Identification: A 1-cm. layer of a 0·0025 per cent w/v solution in water exhibits characteristic light absorption with maxima at 269, 298 and 354 mμ.

Light absorption: The extinction of a 1-cm. layer of a 0·0025 per cent w/v solution (freshly prepared) in water, calculated for the anhydrous material, at 298 mμ is about 0·55 and at 354 mμ about 0·61; the ratio of the extinction at 354 mμ to that at 298 mμ is greater than 1·0.

Acknowledgements. The authors wish to thank Miss M. D. Bradley and Mr. J. A. Grant for technical assistance. Thanks are due also to Mr. D. R. Wood for help with analyses, to Dr. E. S. Stern and Professor L. J. Haynes for advice.

References

1. Paris and Durand, *Ann. pharm. franç.*, 1956, **14**, 755.
2. Auterhoff and Ball, *Arzneimit.-Forsch.*, 1954, **4**, 725.
3. Latven, Sloane and Munch, *J. Amer. pharm. Ass. Sci. Ed.*, 1952, **41**, 548.
4. Hay and Haynes, *J. chem. Soc.*, 1956, 3141.
5. Böhme and Bertram, *Arch. Pharm.*, 1955, **288**, 510.
6. Hagedorn, *Dtsch. Apoth. Ztg.*, 1952, **92**, 985.
7. Jaminet, *J. pharm. Belg.*, 1957, **12**, 87.
8. Awe, Auterhoff, Wachsmuth-Melm, *Arzneimit.-Forsch.*, 1958, **8**, 243.
9. Markham and Smith, *Nature, Lond.*, 1949, **163**, 250.

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

A microscopical means of differentiating between crystalline and amorphous aloin was to mount the material in cresol, when the amorphous material was soluble while the crystalline was not.

SOME ASPECTS OF THE STORAGE AND TESTING OF STERILISED CATGUT

By G. R. Wilkinson, E. L. Robins, J. J. Grimshaw and S. P. A. Hudson

From the Research Division, Allen & Hanburys Limited, Ware, Herts.

Received May 21, 1959

Results obtained during an examination of plain and chromicised surgical catgut stored in tubing fluids of differing water content, and the effect on diameter, tensile strength and flexibility are described. A fall in tensile strength and an increase in diameter which vary with water content and time of storage was observed, although gut does not deteriorate rapidly when immersed in water and several days elapse before significant changes occur. The official testing methods have been examined, and the results indicate that the surgeon's knot test gives a more reliable measurement of tensile strength than the straight pull. Also the present gauge method for measurement of diameter can lead to errors due to compression of the gut. The time of equilibration of the gut in air is important for the measurement of both tensile strength and diameter, particularly when tubing fluids contain a high water content. A relation has been shown between tensile strength, extensibility and softening, and it is suggested that this might provide an index of flexibility of the gut. The effect of some common disinfectant solutions on the gut has been investigated.

The first monograph on surgical catgut in an official publication in the United Kingdom appeared in the B.P.C. Supplement VI (1944) and this monograph has remained substantially the same to the present day and now appears in the B.P. 1958.

The preparations are packed in a wide variety of "tubing fluids" of varying composition[1–10] for which advantageous properties, like enhancement of flexibility, are claimed.

Douglas[11] (1949) criticised the official methods of test on the grounds that the figures obtained for tensile strength were not realistic.

This paper reports the results obtained during a storage test on catgut and an examination of the testing methods and comments upon them.

Experimental

The number of variables was reduced to reasonable proportions by selecting the two most used grades of catgut, namely 2/0 Plain and 2/0 Chromicised Medium Hard.

Strands were selected so that their diameters were nearly uniform and were about the mean of the prescribed limits. These strands were subjected to dry sterilisation and subsequent tests showed them to be sterile.

Cut into lengths of 15 inches the gut was randomly distributed into fluids contained in 2 litre glass-stoppered cylinders so that the proportion of fluid per strand was about the same as when packed in tubes. Further samples were sealed in tubes containing similar fluids. The cylinders were stored at room temperature, to simulate normal storage and the sealed tubes at 37°, as an accelerated test to provide advance information of possible changes at room temperature.

TABLE I

COMPARISON OF OPTICAL AND MICROMETER METHODS FOR THE MEASUREMENT OF THE DIAMETER OF CATGUT

Water concentration, per cent	Chromic gut		Water concentration, per cent	Plain gut	
	Optical	Gauge		Optical	Gauge
Controls	36·12	35·29	Controls	38·25	36·98
5	38·12	36·60	5	36·50	34·77
25	38·56	38·04	10	36·96	35·58
50	36·88	35·58	50	43·21	35·17

B.P. Limits 31·8 to 40·6 mm./100

ANALYSIS OF VARIANCE (CHROMIC GUT)

Source	S.S.	d.f.	M.S.	F.	Significance
Between methods	101·0651	1	101·0651	22·98	S.
Between groups	391·82031	3	130·60677	29·69	H.S.
M × G	13·19532	3	4·39844	1·43	N.S.
Strands within groups	2665·61459	44	60·58214	16·88	V.H.S.
S × M	157·86458	44	3·58783	1·16	N.S.
Between strands	3329·5599	95			
Residual	888·25	288	3·0842		
Total	4217·8099	383			

TABLE II

CHANGE IN DIAMETER OF CATGUT DURING EQUILIBRATION PERIOD

	Optical		Gauge	
	Mean	s.e. Mean	Mean	s.e. Mean
Control chromic R.T. 11 months				
0 min.	36·54	1·810	37·38	1·991
5 min.	38·95	0·567	38·06	0·598
10 min.	35·39	0·652	34·25	0·637
15 min.	36·32	0·194	33·69	0·120
20 min.	36·99	1·480	35·94	0·409
100 per cent H_2O chromic R.T. 11 months				
0 min.	47·48	1·648	41·38	1·897
5 min.	38·72	0·532	36·31	0·187
10 min.	38·13	1·449	37·12	0·486
15 min.	31·53	0·541	31·62	0·681
20 min.	35·28	0·862	35·75	0·889
Control plain R.T. 11 months				
0 min.	43·64	0·174	40·19	0·360
5 min.	38·13	1·224	38·94	0·624
10 min.	37·69	1·949	37·38	1·615
15 min.	35·29	1·002	34·56	1·183
20 min.	37·47	0·391	34·50	0·685
100 per cent H_2O plain R.T. 11 months				
0 min.	52·34	1·586	32·75	1·237
5 min.	45·44	1·515	31·31	1·484
10 min.	34·25	1·611	30·88	1·379
15 min.	36·88	1·749	33·69	1·378
20 min.	35·18	0·922	35·19	0·832

The fluids had the following composition. (1) Potassium mercuri-iodide 0·2 per cent in industrial methylated spirit (96 per cent). (2) Fluid 1 with 5 per cent v/v added sterile distilled water. (3) Fluid 1 with 10 per cent v/v added sterile distilled water. (4) Fluid 1 with 25 per cent v/v added sterile distilled water. (5) Fluid 1 with 50 per cent v/v added sterile distilled water. (6) Potassium mercuri-iodide 0·2 per cent in sterile distilled water. (7) Sterile distilledwater.

Examinations were made before the test commenced ($t = 0$), after 3, 7, 30, 60, 90 and 180 days, and sufficient samples were retained at room temperature for examination at 360 days.

TABLE III

EFFECT OF EQUILIBRATION ON THE TENSILE STRENGTH AND EXTENSION OF CATGUT

			Time (min.) 0	15
Tensile strength S.P. (lb.) ..	Control	Mean	6·54	7·33
		s.e. mean	±0·726	±0·446)
		t	<1·812	
		P	>0·1	
		Significance	N.S.	
	100 per cent water	Mean	4·38	6·08
		s.e. mean	±0·248	±0·601
		t	2·628	
		P	0·05 > P > 0·02	
		Significance	S.	
Extension (in.	Control	Mean	1·46	1·46
		s.e. mean	±0·070	±0·053
		t	0	
		P	—	
		Significance	—	
	100 per cent water	Mean	1·40	1·33
		s.e. mean	±0·099	±0·095
		t	<1·812	
		P	>0·1	
		Significance	N.S.	

Plain gut after 1[illegible] months at room temperature was used throughout. The tensile strength was assessed by the straight pull test, and the extension was of 5 inch strands up to the break-point.

All measurements were made according to the B.P. 1958 monograph.

On each 15-inch length one determination of tensile strength was made by a straight pull and a second over a surgeon's knot, and the results subjected to statistical analysis.

Diameters were determined at approximately equal intercepts along each 15-inch strand, in the early stages of the experiments by the micrometer method and later optically in addition, because contrary to expectations, there appeared to be a reduction in diameter.

Using the dial gauge micrometer of the type suggested in the pharmacopoeias, considerable pressure is found to be exerted on the gut. Although the pressure on the whole of the anvil averages only 200 g. (1·625 kg./sq.cm.), the pressure exerted by the pressor foot on the gut, assuming a line contact of about one-quarter of its diameter for the 2/0 gauge, is in excess of 160 kg./sq.cm. This high pressure is responsible for an apparently smaller diameter compared with the results obtained when pressure is not used and is variable according to the thickness of the gut and its softness.

The optical method used a microscope eyepiece micrometer calibrated against pieces of wire of different gauges about the range under consideration.

The gut when measured optically does not always conform to the B.P. specification for diameter, whereas when measured by the micrometer falls within these limits. Table I summarises the salient figures, which have been abstracted from the 180 days storage data and an analysis of variance for chromic gut is given.

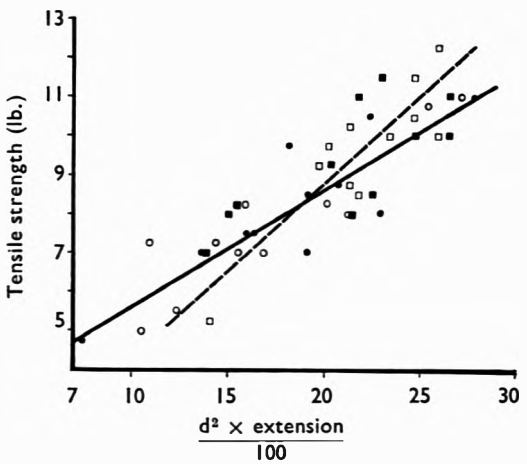

FIG. 1. The relation between extension, cross sectional area and tensile strength of surgical catgut stored at room temperature for 11 months. Tensile strengths were measured by the straight pull test. Extension was measured in inches up to the break-point. Diameters were measured optically.

○ = Plain gut in Fluid 1 (control).
● = „ „ 7 (100 per cent H_2O).
□ = Chromicised gut in Fluid 1 (control).
■ = „ „ „ 7 (100 per cent H_2O).
— Plain gut. — — Chromicised gut.

Owing to the implications of these differences a special test was introduced in which further comparisons were made, and these substantiated the findings particularly where swelling of the gut had occurred because of the high water-content of the fluid.

As an example, the diameter in hundredths of a millimetre of plain gut in fluid 6 (100 per cent water) were 33·31 (micrometer), 36·61 (optical) at 180 days and analysis of variance showed this difference to be highly significant.

A comparison between the B.P. and U.S.P. methods of test revealed a major difference in detail. The B.P. requires a time lapse of 10 to 15 minutes in free air between removal of the strand from the fluid and determination of the physical characteristics of the gut, whereas according to the U.S.P. the determinations are made immediately upon removal from the fluid. This feature has been examined by measuring the diameter of gut removed at 180 days from fluid 1 (control) and fluid 6 at times up to 20 minutes (Table II). This shows the differences between the results obtained by the two methods until the gut has dried sufficiently to resist the pressure applied to it in certain groups. These results are confirmed in other experiments.

Since modern tubing fluids may contain a proportion of water or other materials for the express purpose of keeping the suture in a flexible condition it is still possible even after 10 to 15 minutes drying for the gut to be sufficiently flexible to be distorted by pressure.

As different diameters were recorded according to the time of equilibration in air before measurement, the tensile strength was also determined at 0 and 15 minutes after removal from the fluid and the results are given in Table III, which gives figures for the straight pull tests during which extension was measured.

A significant increase in tensile strength was recorded for gut in fluid 6 after 15 minutes compared with time 0. With fluid 1 (control) the increase in tensile strength was not significant.

To examine the relation between the extension and the tensile strength of the gut an attempt was made to apply Young's Modulus and the plot of the results obtained for plain and chromicised gut stored for 11 months at room temperature is given in Figure 1. Good correlation ($r = 0{\cdot}86$) was obtained. Similar results were obtained with chromicised gut ($r = 0{\cdot}80$). There was a direct relation between the water content of the fluid and extensibility of chromic gut. Analysis of variance showed the relation to be quadratic and the line of best fit in Figure 2 has been calculated by the method of least squares.

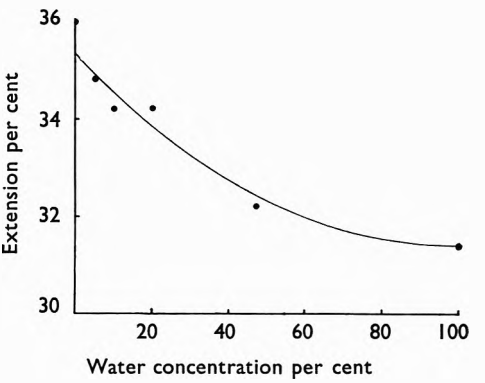

FIG. 2. Relation between extension measured to the straight-pull break-point and the water concentration of the tubing fluid.

An attempt was made to correlate extensibility and softening. As already mentioned, there was a significant difference in optical and gauge diameters when gut was measured immediately after removal from fluid which disappeared after 15 minutes equilibration.

DISCUSSION

The data presented largely confirms the belief that there is a deterioration in surgical catgut when stored for long periods in high concentrations of water but contrary to some opinion there is no rapid change even in 100 per cent water. In all concentrations of water chromicised gut is considerably more stable than plain and storage at 37° accelerates deterioration.

In tubing fluids of high water content where swelling occurs there can be a large difference between diameters measured by micrometer gauge and when pressure is not applied. The gauge can suggest a reduction in diameter due to softening, whereas in fact the gut has increased in diameter. This effect varies with the water content and with the type of gut, and disappears only after 15 minutes equilibration, so if the measurements are made in the B.P. and U.S.P. periods of less than 15 minutes erroneous results will be obtained.

In the analysis indicated in Table IV an attempt has been made to relate softness and flexibility. The ratio of optical to micrometer gauge measurement of diameter, a measure of softness, has been related with the extension of the gut up to the break-point. A high negative correlation exists when measurements are taken as soon as the gut is removed from the tubing fluid but since the optical to gauge ratio decreases to unity after 15 minutes equilibration the correlation becomes insignificant at this time. The correlation coefficient is larger in the gut stored in fluid 6 than in fluid 1 (control) both at time 0 and after 15 minutes. For

TABLE IV

THE EFFECT OF EQUILIBRATION ON RELATIONSHIP BETWEEN OPTICAL/GAUGE DIAMETER RATIO AND EXTENSION TO BREAK POINT

Control 11 months R.T.			
Time zero		15 minutes	
O/G	Extension (in.)	O/G	Extension (in.)
0·934	1·625	0·929	1·375
0·992	1·5	0·938	1·5
0·998	1·5	0·969	1·625
1·029	1·5	0·971	1·25
1·040	1·5	0·972	1·5
1·076	1·125	0·976	1·5
$r = -0.814$		$r = 0.156$	

100 per cent water 11 months R.T.			
Time zero		15 minutes	
O/G	Extension (in.)	O/G	Extension (in.)
1·308	1·75	0·932	1·75
1·340	1·625	0·978	1·25
1·385	1·375	0·987	1·125
1·404	1·25	0·989	1·125
1·420	1·25	1·004	1·375
1·491	1·125	1·100	1·375
$r = -0.953$		$r = 0.269$	

Plain gut was used throughout. Diameters were measured at 4 equal intercepts, and extension to the break-point, on each strand. O/G = Ratio of optical to micrometer gauge diameter.

measurement of tensile strength, the surgeon's knot test provides a more reliable indication of the quality of the gut than does the straight pull test. Furthermore, since the limits for the tensile strength by the surgeon's knot test given in the B.P. are exactly half for the straight pull and in the U.S.P., where the knot is tied round a $\frac{1}{4}$ inch diameter rubber tube, about 70 per cent, there appears little reason for inclusion of the straight pull test. As in an interrupted suture, which, according to Douglas[11], is the weakest kind, a knot is always tied, it appears logical for the gut to be thus tested.

The B.P. requires two determinations on a strand exceeding 30 inches in length, the lower reading to be taken as the breaking load, and for this figure to be considered in relation to stated acceptance limits. The U.S.P. takes the mean without attempting to consider variation within the batch. These are crude systems of quality control, the B.P. method

being better than the U.S.P., as with the latter it is possible for figures as wide apart as 0·25 and 10 lb. on two strands to be averaged and to pass the test for 2/0 gut. By a consideration of all the figures, however, it is possible to obtain a measurement of variation within the batch and this could be used to provide a more rigorous quality control scheme.

The difference between the methods of testing in the U.S.P. and B.P. monographs concerning equilibration may have a considerable effect if tubing fluids of high water content are used (see Table III where the straight pull test is illustrated) but in the surgeon's knot test the effects are at a minimum.

We have also examined the ratio of "surgeon's knot strength/diameter^2" for each sample of control and purchased gut in an attempt to eliminate differences due to unequal matching of diameters and in all cases the control gut showed to advantage.

Since catgut in use may come into contact with some common disinfectants in which tubes of gut are stored in hospitals and which are used in the operating theatre, it is interesting to note that those of the phenolic type have no effect on the gut. This is important as it is well known that phenol, in particular, can seep through minute cracks in glass tubes.

There is no provision made in either the B.P. or the U.S.P. for a standard for flexibility, although from the surgeon's point of view this property ranks in importance with tensile strength.

It is suggested that the test for extensibility described provides an assessment of the flexibility of the gut.

Other workers[12] have shown that catgut under longitudinal stress has similar properties and behaves in a similar manner to metallic wires, an X-ray diffraction pattern indicating an amorphous structure in the resting state and re-orientation into crystalline micelles when under stress. We have shown that for a given type of gut extensibility and tensile strength both change on storage, but Figure 1 shows that Young's modulus remains constant; that is, the plot of tensile strength against cross-sectional area $\times$ extension is coincidental for gut stored in fluid 1 (control) and fluid 7.

Since it would appear that plain and chromicised gut have much the same physical characteristics, we fitted a common regression line to both sets of data and obtained $r = 0{\cdot}87$ for the correlation. Analysis of variance for the regression of tensile strength on the product of cross-sectional area and extension showed a very highly significant linear regression. This confirms that catgut however treated maintains a fundamentally characteristic relationship between its flexibility and its tensile strength.

The relationship between the surgeon's knot and straight pull was also examined but no relation was obtained between this ratio and water concentration, elongation or optical to gauge ratio. A limitation of the work is that gut of only one gauge was used to reduce the variation. This automatically reduced the diameter range but, nevertheless, within the working range there was sufficient variation for the more important relationships to be demonstrated. It is possible that other effects would have been found had a wider range of gut been used.

Acknowledgements. Thanks are expressed to T. Barr, C. Davis and G. A. McCormick, who assisted in obtaining and presenting the information for this paper, to Miss J. Hancox and Miss B. Barnwell for the computation and to Miss F. McKechnie for a literature search.

References

1. Tubing Fluids for Catgut, *J. Amer. Pharm. Ass.* (Pract.), 1948, **9**, 49.
2. Catgut Sutures, Powers and Ayres, U.S. Patent 2,694,487; 16.11.54, *Chem. Abstr.*, 1955, **49**, 3481.
3. Suture Package, Davis and Geck Inc., B. Patent 751,331; 27.6.55.
4. Suture Packages, Ethicon Suture Laboratories, B. Patent 753,293; 19.7.56.
5. Improvements Relating to Surgical Suture, Davis and Geck Inc., S. Africa application 2709/54; 26.4.54.
6. Preserving Suture Material Sterile before and during use, Gelinsky, E., U.S. Patent 2,128,701; 30.8.38, *Chem. Abstr.*, 1938, **32**, 8708.
7. Sutures, Davis and Tringali, U.S. Patent 2,524,772; 10.10.50, *Chem. Abstr.*, 1951, **45**, 830.
8. Tertiary-Butyl Alcohol as a Fluid, Ayres G.B., U.S. Patent 2,739,704, 27.3.56 *Chem. Abstr.*, 1956, **50**, 8973.
9. Mixed Alcohols as Tubing Fluids for Catgut, Banks R.M., U.S. Patent 2,742,148; 17.4.56, *Chem. Abstr.*, 1956, **50**, 15032.
10. Non-boilable tubing fluid for sutures, Runkel and Zielinski, U.S. Patent 2,796,984; 25.6.57, *Chem. Abstr.*, 1958, **52**, 1556.
11. Douglas, *Lancet.* 1949, **2**, 497.
12. Clark, Flege, Ziegler, *Industr. Engng Chem.*, 1934, **26**, 440, *Chem. Abstr.*, 1934, **28**, 3521.

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

Measurements were made using a microscope eyepiece micrometer without tension applied to the strings and with a check for roundness; as far as possible the dial guage was applied at the same point. The tensile strength and total extension to break point were measured using a B.P. type apparatus without studying the pattern of the extension. Tying a knot on a piece of rubber tubing apparently increases the strength. The effect of methanol on flexibility and diameter had not been overlooked, but water was the factor related to pliability, and measurements made during the first 72 hours' storage were without significant results.

Short Communication

THE ABSORPTION AND DISTRIBUTION OF HALOTHANE

By W. A. M. Duncan

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Received May 27, 1959

Halothane (Fluothane), 1, 1, 1-trifluoro-2-chloro-2-bromoethane, the pharmacology of which was studied by Raventos[1], is a non-explosive volatile anaesthetic which was first used clinically by Johnstone[2].

The absorption and excretion of ether and chloroform were studied early in this century, and since then similar studies have been carried out on the other volatile anaesthetics. These studies showed that the depth of anaesthesia is dependent upon the concentration of the anaesthetic in the arterial blood and in the central nervous system, and that this is controlled by the concentration of the agent in the inspired gas mixture.

Goodall[3] described a method for the analysis of halothane in which a light petroleum extract of blood from an anaesthetised animal was heated with sodium amoxide at an increased pressure for 2 hours. Some 60–80 per cent of the bromine of halothane was liberated. It was later found that halothane could be degraded at room temperature with an ether solution of lithium aluminium hydride which, in 20 minutes, liberated 85 per cent of the bromine and 30 per cent of the chlorine[4]. The liberated halide was then extracted and estimated nephelometrically by the addition of silver nitrate. This method was suitable for routine use in the estimation of halothane in blood and tissues.

Mice and rats were anaesthetised in an atmosphere of usually 1·5 per cent v/v halothane in oxygen, without any premedication, using the apparatus which has been described by Raventos[1]. The kinetics of halothane anaesthesia and the distribution of the agent in the tissues were studied by exposing the mice, or rats, for varying lengths of time to the anaesthetic after which the animal was killed and the halothane content of the whole mouse or the separate tissues of the rat determined. In a similar way the concentration of halothane in the whole animal, or in the tissues, at different times after the cessation of inhalation of the agent was examined.

It was found that in the mouse halothane was rapidly absorbed during the first 10 minutes of inhalation of the anaesthetic; after 10 minutes, a 20 g. mouse contained about 6 mg. of halothane. As the duration of anaesthesia increased the absorption of the agent continued more slowly so that after 180 minutes anaesthesia a 20 g. mouse contained about 35 mg. of halothane. This was the longest period of anaesthesia studied in the mouse but, as there was no decrease in the rate of absorption it is obvious that the tissues would be able to absorb considerably more halothane. On recovery from anaesthesia with halothane, which had been administered for 2 hours, it was found that the total amount of

anaesthetic in the animal decreased rapidly; the half-clearance time was about 30 minutes.

In the rat, the rates of absorption of halothane by the arterial blood, the brain, the liver and the perirenal fat were examined and several unexpected facts observed (Fig. 1). All of these tissues showed an initial rapid absorption of the agent, similar to that found in the whole mouse. The perirenal fat was found to have the greatest affinity for the agent, which was not surprising as halothane has a high oil : water partition coefficient. However, even after 6 hours anaesthesia, at which time the fat contained about 1 per cent by weight of halothane, there was no indication of the saturation of this tissue with the agent. The liver and brain gave identical results; this was attributed to the poor solubility of halothane in phospholipids and to the difference in the blood supply in the two tissues. The concentration of halothane in the brain was about 45 mg./100 g. after 6 hours. This slow increase in the concentration of halothane in the brain contributes to the small influence which the duration of anaesthesia has on the time taken to recover after anaesthesia. The arterial blood was observed to reach a state of equilibrium with the inhaled gas concentration in about 90 minutes, when the concentration of halothane was about 20 mg./100 ml. of blood. This is quite different from what has been observed with ether and chloroform where the concentration of the agent in the blood increases with continued anaesthesia.

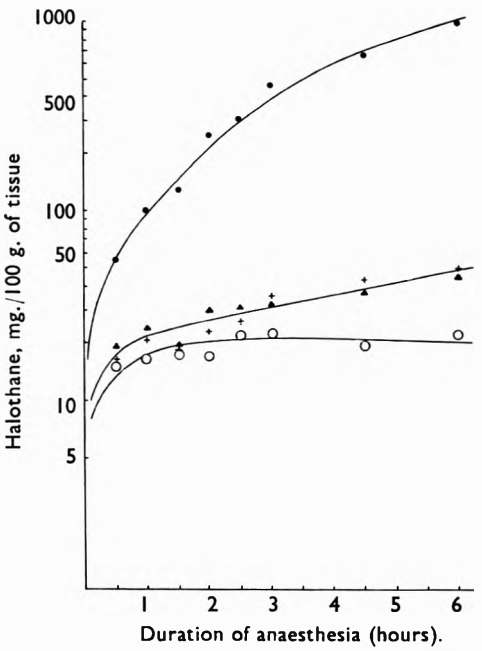

FIG. 1. The absorption of halothane by the tissues of rats during anaesthesia with 1·5 per cent v/v halothane in oxygen. ○—○ Arterial blood; +—+ liver; ▲—▲ brain; ●—● perirenal fat.

During the period following cessation of inhalation of halothane a very marked difference was observed between the arterial and venous blood systems in the rates of elimination of the anaesthetic (Fig. 2). The agent was cleared rapidly from the arterial blood, the half-clearance time being about 15 minutes, whereas the rate of clearance from the fat and the venous blood was slower with a half-clearance time for both of 45 minutes.

In man it was observed that for anaesthesias lasting more than 30–60 minutes, the half-clearance time of halothane from the venous blood was about 25 minutes.

The half-clearance time of an inert gas from the body is a function of the blood: gas partition coefficient of the gas. Butler[5] derived the following expression:—

$$\text{Half-clearance time (min.)} = \ln 2 \frac{V_D(V_P + \lambda C_P)}{V_P \cdot C_P}$$

Where V_D = volume of distribution of the drug in litres
V_P = pulmonary ventilation in litres per minute
C_P = pulmonary circulation in litres per minute
and λ = blood: gas partition coefficient.

With the following values $V_D = 70$ litres, $V_P = 8$ litres per minute, $C_P = 5$ litres per minute and with the appropriate blood: gas partition coefficient (halothane 3·6, chloroform 7·3, and ether 15) it can be calculated that the half-clearance time of halothane is 31, of chloroform 54 and of ether 100 minutes.

The calculated value for halothane is in close agreement with those reported above and thus substantiates the view that the blood: gas partition coefficient is one of the main controlling factors in the exhalation of a volatile anaesthetic. Halothane has a low blood: gas partition coefficient and therefore during recovery the anaesthetic is almost completely cleared from the venous blood during its passage through the lungs so that no "reanaesthetisation" of the patient, or animal takes place; this does occur with ether which has a much higher blood: gas partition coefficient. The gas or volatile anaesthetic which is rapidly exhaled during recovery is also readily absorbed during induction so that the onset of anaesthesia with halothane is quick.

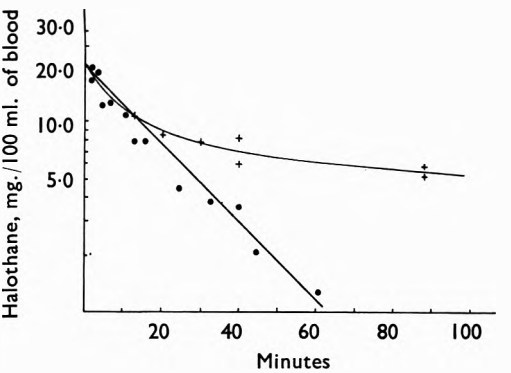

FIG. 2. The elimination of halothane from the arterial and venous blood of rats after anaesthesia with halothane. ●—● Arterial blood; +—+ venous blood.

The correlation of the calculated and experimentally determined half-clearance times of halothane indicate that it is not extensively metabolised. Several different experiments were carried out to determine whether halothane was metabolised; all gave negative results.

REFERENCES

1. Raventos, *Brit. J. Pharmacol.*, 1956, **11**, 394.
2. Johnstone, *Brit. J. Anaesthesia*, 1957, **29**, 135.
3. Goodall, *Brit. J. Pharmacol.*, 1956, **11**, 409.
4. Duncan, *Brit. J. Anaesthesia*, In the press.
5. Butler, *Fed. Proc.*, 1958, **17**, 1158.

After Dr. Duncan presented the communication there was a DISCUSSION.

AN INDICATOR CONTROL DEVICE FOR ETHYLENE OXIDE STERILISATION

By A. Royce and C. Bowler

From the Microbiology Division, Standards Dept., Boots Pure Drug Co. Ltd., Nottingham

Received May 22, 1959

The use of physical sterilisation controls instead of bacteriological controls for heat sterilisation processes is cited. Due to sorption of the gas and penetrability difficulties added need for such controls exists in ethylene oxide sterilisations. Some data on sorption of ethylene oxide is presented. The construction of a simple disposable device, to indicate the attainment of lethal conditions in ethylene oxide sterilisations is described. Data are presented to compare the control device with bacteriological controls, under different conditions of time, temperature, and gas concentration.

The classic method of testing the effectiveness of any microbial sterilisation is to include in the sterilisation a suitable preparation of resistant organisms. Subsequently the preparation is cultured to ascertain whether the organisms were killed. Such a process is time-consuming, some days must elapse before even a reasonably certain answer can be given. It also requires specialised laboratory staff. In addition there are considerable difficulties in obtaining suitable resistant organisms, and in maintaining their resistance.

In the field of heat sterilisation many attempts have been made to produce devices which are suitable routine controls on sterilisers and which will give an immediate indication on completion of the process that it was effective. These range from various melt tubes and colour change papers, which indicate merely that a certain temperature was reached or exceeded momentarily, to more suitable devices which do indicate whether a certain time at temperature was exceeded or not.

This paper describes the extension of the "physical control" to gas treatments in general, and to ethylene oxide sterilisations in particular.

Ethylene oxide has been used for more than 20 years as an insecticidal fumigant and as a sterilising agent against micro-organisms. Its initial use was covered by various patents[1–5], but considerable disagreement as to the mode of usage was evident, in that some specified that moisture must be present whilst others that it should be absent.

It was not until the work of Phillips and Kaye[6–8] in 1949, that it had very much application as a sterilising agent against micro-organisms. Subsequently papers by Velu and others[9] 1942, Roberts and others[10] 1943, Royce and Sykes[11] 1955, Rausher and others[12] 1957, and Grundy and others[13] 1957 all described its use and application to many sterilisation purposes but again there was considerable variation in the gas concentrations, time of exposure and temperatures specified.

Whilst some of this confusion may be due to the "partly protected" state of organisms in various substances treated, and to variations in

humidity, much is probably due to sorption of the gas by the materials being treated.

Although in its application to insecticidal fumigation[14] and in other connections[15] the sorption of ethylene oxide was well understood, there are few references in the field of sterilisation to sorption effects. Since these questions of sorption were largely the stimulus prompting the production of the sterilisation control described later, we have included a selection of sorption data which illustrates the wide differences between different materials and products. In presenting this we have attempted to relate it quantitatively to practical sterilisation conditions (Table I).

In any practical sterilisation, the amount of ethylene oxide absorbed depends upon the gas concentration and the amount of sorptive materials

TABLE I

SORPTION OF ETHYLENE OXIDE BY VARIOUS MATERIALS

Material	Amount of ethylene oxide sorbed after 18 hr. contact with 10 per cent v/v ethylene oxide in air at room temp. (20° C. approx.) mg./g.
Polythene	2
P.V.C.	19·2
Bakelite	Nil
Brown paper	6·1
Cardboard	10·4
Packing case wood	18·4
Cotton wool (absorbent)	3·5
Cotton wool (non-absorbent)	4·1
Red rubber closures	5·5
White rubber closures	7·4
Black (neoprene) rubber closures	15·2
Starch glove powder	10·5
Kaolin	2·5
French chalk	0·5
Sulphanilamide	0·8
Procaine penicillin	0·2
Water	25–30

present in the steriliser. Given sufficient time a state of equilibrium between the ethylene oxide concentration in the atmosphere of the steriliser and in the materials being treated will eventually be reached. The time required to reach this point depends on the nature of the absorbing material, its thickness, physical state, surface area, etc., and the temperature.

The death of an organism depends amongst other things on the gas concentration surrounding it, temperature, and time. Thus, if gas is sorbed by material in the steriliser, the concentration available to kill organisms is lowered and a consequently longer time will be required.

EXPERIMENTAL

When micro-organisms are exposed to lethal gases, the time required to kill the organisms at any given temperature varies inversely with the gas concentration, provided the organisms are not "protected" in some way from the gas, and provided that the conditions of relative humidity are appropriate. This lethal time-concentration product is roughly constant, over a wide range of gas concentrations; it varies inversely with temperature, a smaller one being lethal at higher temperatures, and a greater one being required at lower temperatures.

This has a close parallel in the diffusion of a gas through a permeable membrane. Here also the time required for a given quantity of gas to diffuse through a given area is inversely proportional to the gas concentration. Similarly at higher temperatures the diffusion rate is increased and at lower temperatures is reduced.

If therefore a quantity of a suitable absorbent for the gas containing a suitable indicator is enclosed in a gas-permeable envelope or sachet of controlled dimensions, then by variation of the quantity or strength of absorbent, or the dimensions of the sachet, it is possible to make an "artificial organism" which will change colour after the absorption of a certain quantity of gas. If it can also be arranged that this change takes place after the absorption of a lethal time-concentration product of the gas, a control is produced, which when included in a gas sterilisation, will indicate whether lethal conditions were attained at that place in the steriliser. Such indication would of course be apparent immediately on

TABLE II

TIME REQUIRED TO KILL SOIL DUST SPORES OR TO CHANGE THE SACHET CONTROL AT CONSTANT TEMP. (20° C.) IN VARYING CONCENTRATIONS OF ETHYLENE OXIDE

Ethylene oxide concentration per cent v/v in air or nitrogen	Time in hours required to	
	Sterilise soil dust containing 10^6 spores/g.	Change colour of sachet control, yellow to purple
100	<4	2
50	4–7	4
25	7–8	8
10	16	19
5	24	45
2·5	48	95

completion of the sterilisation and would require no further laboratory work. For ethylene oxide a suitable absorbent is a saturated aqueous solution of magnesium chloride containing hydrochloric acid[16]. This solution quantatively absorbs the gas to form ethylene chlorhydrin, the reaction of the solution becoming more alkaline.

Ethylene oxide is frequently employed in gas sterilisation processes mixed with large quantities of carbon dioxide to render the mixture non-explosive[17]. It is therefore necessary to use an indicator that is insensitive to carbon dioxide. There are many such indicators, we have chosen to use bromophenol blue.

Most plastics are readily penetrable by ethylene oxide and the envelope can be made of polythene, P.V.C. or nylon film, no doubt others could also be used. We have chosen for small scale work to use polythene. It is possible to make up envelopes of any dimensions from sheet material, but since "lay-flat" tubing in a great variety of widths and thicknesses is readily available it is more convenient to use this.

Since considerable divergence of view exists on the question of what constitutes a lethal time-concentration product for ethylene oxide, we have chosen to make these devices on the basis of our own work on ethylene oxide sterilisation. We have found over a period of years that a 10 per cent v/v gas concentration (200 mg./l.) for a time period of 16 to

18 hours at ambient temperatures is a convenient practical way of employing ethylene oxide. This process is based on the sterilisation of soil dust spores, which are admittedly difficult to sterilise. The devices can be manufactured to yield a positive result at any other time-concentration product if desired. The technical details of the device produced to control the practical sterilisation process outlined are included in the Appendix 1.

Tests were made with the devices in varying concentrations of ethylene oxide at a constant temperature (20°). Bacteriological soil dust spore preparations were also exposed to similar gas concentrations at the same temperature. These tests are summarised in Table II. A further series of tests was made using a constant concentration of ethylene oxide, at

TABLE III

TIME REQUIRED TO KILL SOIL DUST SPORES OR TO CHANGE THE SACHET CONTROL, IN A CONSTANT CONCENTRATION OF ETHYLENE OXIDE (10 PER CENT V/V), AT VARYING TEMPERATURES

Temperature ° C.	Time in hours required to	
	Sterilise soil dust containing 10^6 spores/g.	Change colour of sachet control, yellow to purple
10	32	32
20	16	19
30	7	9
40	5	6·5
50	4	4·5
60	3	3

different temperatures. Summarised results of these tests on soil dust spores and the sachet controls are given in Table III. Various tests with the devices have shown that they are sensitive to a concentration time product difference of approximately 10 mg. hr./l. This represents a difference of less than 0·5 per cent in the time-concentration product used in the sterilisation process quoted. Other slight variations occur in use, due to diffusion and mixing rates inside the sachet, and to slight dimensional differences in the sealed sachets. The sum of all such differences however does not introduce error greater than $\pm$ 2·5 per cent. This is considerably less than is tolerable for a steriliser control, and is certainly much smaller than the errors inherent in bacteriological controls.

DISCUSSION

It is evident that irrespective of wide differences in gas concentration, time of exposure and temperature, change of colour of the sachet control indicates that a lethal combination of these variables to micro-organisms existed at that point in a steriliser.

Due to sorption of the gas almost any ethylene oxide treatment must be a treatment with a varying gas concentration, and a control of the type described provides a means of integrating the total effect of such a treatment which would be difficult to achieve by other means. This is particularly so when one considers the different rates of diffusion and penetration of the gas into different types of materials and packages,

producing many differing concentrations, all changing at different rates, at various points in the materials being treated, in a particular sterilisation.

Whilst "blunderbuss" methods of greatly increased concentrations could no doubt resolve some of these difficulties, such methods are frequently self-defeating in that the increased concentrations cause increased sorption to occur. Additionally, deleterious change in the materials being treated is made more likely, and of course the cost of treatment is increased. A means of measuring the actual effect of the process at any point with a fair degree of accuracy can be a very valuable tool, in designing or evaluating sterilisation processes.

The principle can be applied to many other gases and to gas treatments other than sterilisation treatments. In the sterilisation field we have produced similar devices for formaldehyde treatments, and in the related field of insecticidal fumigation devices to control methyl bromide as well as ethylene oxide have been made. The applications to insecticidal fumigations will form the subject of another paper, which will be published elsewhere. Patent applications have been filed, and various types of these controls are now available.

Appendix

Specification for Control Sachet suitable for 10 *per cent v/v Ethylene Oxide Sterilisations at* 20° *for* 16 *to* 18 *hours*

Polythene "lay-flat" tubing of 0·005 in. thickness and 1 in. width is used.

This is cut into lengths of approx. 2¼ in. so that when both cut edges are sealed the finished sachet has a length of 2 in. One cut edge is then heat sealed. 5 ml. of the absorbent solution is introduced into the envelope.

The absorbent solution is saturated aqueous magnesium chloride solution containing 0·004 per cent bromophenol blue, acidified with hydrochloric acid so that 5 ml. of the solution is equivalent to 3 ml. 0·1 N HCl using bromophenol blue as indicator.

The other edge is heat sealed.

References

1. Schrader and Bossert, 1936, U.S. Patent 2,027,439.
2. Gross and Dixon, 1937, U.S. Patent 2,075,845.
3. Griffith and Hall, 1940, U.S. Patents 2,189,974 and 2,189,948.
4. Baer, 1941, U.S. Patent 2,229,360.
5. Griffith and Hall, 1943, U.S. Patent Re.22,284.
6. Phillips, *Amer. J. Hyg.*, 1949, **49**, 280.
7. Phillips and Kaye, *ibid.*, 1949, **50**, 270.
8. Kaye, *ibid.*, 1949, **50**, 289.
9. Velu, Lepigre and Bellocq, *Bull. Acad. Med. Paris*, 1942, **126**, 62.
10. Roberts, Allison, Prickett and Riddle, *J. Bact.*, 1943, **45**, 40.
11. Royce and Sykes, *J. Pharm. Pharmacol.*, 1955, **7**, 1046.
12. Rauscher, Mayr and Kaemmerer, *Food Mfg.*, 1957, **32**, 169.
13. Grundy, Rdzok, Remo, Sagen and Sylvester, *J. Amer. pharm. Ass., Sci. Ed.*, 1957, **46**, 439.
14. Pest Infestation Research Annual Report, 1947, H.M.S.O.
15. Royce and Moore, *Brit. J. ind. Med.*, 1955, **12**, 169.
16. El. Khishen, *J. Sci. Food Agric.*, 1950, **1**, 71.
17. Hess and Tilton, *Industr. Engng Chem.*, 1950, **42**, 1251.

After Mr. Royce presented the paper there was a Discussion.

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